Protection of Mice against Philadelphia Chromosome-positive Acute Lymphoblastic Leukemia by Cell-based Vaccination Using Nonviral, Minimalistic Expression Vectors and Immunomodulatory Oligonucleotides


Department of Pediatric Hematology, Children’s Hospital, University of Tübingen, D-72076 Tübingen, Germany [J. K.]; Department of Pediatric Oncology/Hematology, Charité, Humboldt University, Berlin, Germany [J. K., D. B., A. K., H. G. v. E., G. H., K. S.]; Mologen GmbH, Berlin, Germany [S. A. K-M., B. W., M. S.]; Institute for Genetic Medicine, University of Southern California, Los Angeles, California [R. S.]; and Department of Molecular Biology and Bioinformatics, Free University, Berlin, Germany [F. S., B. W.]

ABSTRACT

**Purpose:** Childhood Philadelphia chromosome positive (Ph⁺) acute lymphoblastic leukemia (ALL) has a poor prognosis. Because leukemia cell burden is reduced but not eradicated by polychemotherapy, improved treatment strategies should enhance those immune mechanisms responsible for the maintenance of complete remission. The aim of this study was to evaluate the protection of mice challenged with the syngeneic Ph⁺ ALL cell line BM185 using genetically modified leukemia cell vaccines and immunomodulating oligonucleotides.

**Experimental Design:** Because retroviral vectors are ineffective at transducing nondividing primary cells from human hematopoietic malignancies, we first evaluated nonviral techniques (electroporation and ballistic transfer) using minimalistic immunogenically defined gene expression vectors to generate B7.1 or granulocyte macrophage colony-stimulating factor (GM-CSF)-expressing BM185 cells. Subsequently, protective vaccination experiments with these cells were performed in a leukemia challenge mouse model.

Results: Electroporation yielded a high transfection rate (82.6% for B7.1) with moderate GM-CSF secretion (1 × 10⁶ cells (228 pg), whereas ballistic transfer led to a lower transfection rate (30.9%) with high GM-CSF secretion (614 pg). Secondly, we immunized mice with B7.1/interleukin 2- or B7.1/GM-CSF-expressing BM185 cell vaccines. We observed a better protection of mice that received the B7.1/GM-CSF vaccine compared with those receiving the B7.1/interleukin 2 vaccine. Protection was additionally enhanced by application of a double stem-loop immunomodulating oligonucleotide containing CpG motifs.

Conclusion: Our data indicate that immunization with B7.1/GM-CSF-expressing cell vaccines generated by electroporation and application of double stem-loop immunomodulating oligonucleotide protected mice against a murine Ph⁺ ALL challenge. Ultimately, this approach may also lead to clinical benefit in patients with Ph⁺ ALL.

INTRODUCTION

ALL³ is the most frequently diagnosed malignancy in childhood. In particular, the prognosis of children with ALL and translocation t(9;22)(q34;q11), known as Ph⁺ ALL is still poor (1). In general, the leukemia cell burden in childhood ALL can be reduced but not eradicated completely by polychemotherapy, and the immune system is likely to participate in the elimination of minimal residual disease. Thus, after reduction of leukemia cell burden by chemotherapy, immunotherapy may prevent an ALL relapse by inducing leukemia-specific CTLs.

Although predominantly used for prevention of viral or bacterial infections, vaccines have also been applied recently for the treatment of human cancer (2, 3). This is based on the fact that our immune system, once activated, is capable of developing specific T cell-mediated immunity against neoplastic cells. Lymphoblasts, like most tumor cells, are poor antigen-presenting cells and lack the expression of costimulatory molecules (4). The costimulatory molecule B7–1 triggers a strong T-cell activation through the interaction with the ligand CD28. Interaction of T cells and tumor or leukemia cells without delivery of a costimulatory signal leads to T-cell anergy and apoptosis (5). GM-CSF promotes differentiation and maturation of dendritic cells, and has been shown to enhance the antigen-presenting function (6). The synthesis of IL-2 by tumor cells bypasses the

Received 10/31/02; revised 3/19/03; accepted 3/19/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the Wilhelm-Sander-Stiftung (to J. K. and M. S.), the Deutsche Jose-Carreras-Leukämiestiftung e.V. (to M. S.), and the H. W. and J. Hector Stiftung (to M. S.).

2 To whom requests for reprints should be addressed, at Children’s Hospital, Department of Pediatric Hematology, University of Tübingen, Hoppe-Seyler-Str. 1, D-72076 Tübingen, Germany. Phone: 49-07071-29-87199; Fax: 49-07071-29-5203; E-mail: joachim.koechling@med.uni-tuebingen.de.

3 The abbreviations used are: ALL, acute lymphoblastic leukemia; Ph⁺, Philadelphia chromosome positive; dSLIM, double stem-loop immunomodulator; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MIDGE, minimalistic immunogenically defined gene expression; T helper; ODN, oligodesoxyribonucleotide.
T<sub>H</sub> function and has been shown to induce CTL even in a state of T-cell anergy (7). We and others have shown that vaccination of mice with murine leukemia cell lines retrovirally modified to express costimulatory molecules and cytokines induces a systemic immunity against wild-type leukemia (8–10). However, retroviral transduction is dependent on the proliferation of target cells and, therefore, not appropriate for quiescent primary human ALL blasts. Furthermore, recent reports about induction of leukemia after successful gene therapy of X-linked severe combined immunodeficiency by retroviral gene transfer of the γ<sup>(c)</sup> gene into CD34<sup>+</sup> bone marrow cells raises safety concerns (11). Therefore, nonviral gene transfer methods such as electroporation or ballistic transfer may offer alternative ways to generate genetically modified leukemia cell vaccines. We and others showed previously in clinical trials for treatment of renal cell carcinoma and metastatic colon carcinoma (12, 13), sarcoma (14), and melanoma (13, 15) that autologous tumor cell vaccines generated by nonviral gene transfer were safe and effective.

Regular plasmids include bacterial DNA such as the origin of replication or antibiotic resistance markers, which are known to interfere with transgene expression and which in addition might induce immunological responses toward the vector. For this reason, we have developed a novel MIDGE system (16). The linear vector contains only the essential elements, as there is a promoter, the gene of interest, and a termination site. Both the smaller size of the MIDGE vector and the avoidance of bacterial sequences makes this system more favorable for clinical application than conventional plasmids. We have applied the MIDGE vector system to immunize cats against the feline immunodeficiency virus infection (17) and in a clinical phase I/II vaccine study against metastatic carcinoma (13).

Besides specific immunization by bacterial or viral proteins, the mammalian immune system can be stimulated by defined prokaryotic DNA sequences (18). It has been shown that DNA sequences containing nonmethylated CpG dinucleotides induce B-cell proliferation, and the activation of dendritic cells, natural killer cells, and macrophages (19). In addition, CpG oligonucleotides enhance the expression of MHC class II on dendritic cells and the migratory potential of dendritic cells by reducing E-cadherin-associated adhesion (20), and can directly produce cytokines and macrophages (21). Furthermore, it has been demonstrated that CpG oligonucleotides can be used as therapeutic agents, especially as immunomodulator for vaccines (22, 23).

In view of the poor prognosis of Ph<sup>+</sup> ALL in humans, we used a rather lethal murine Ph<sup>+</sup> ALL challenge model, which has been described in detail previously (10). This model is based on the BALB/c syngeneic pre-B-ALL leukemia cell line BM185 expressing the human BCR-ABL<sup>p185</sup> fusion protein. For analysis of transfection efficiency we first compared nonviral gene transfer strategies such as electroporation and ballistic transfer with retroviral transduction. Secondly, we evaluated the protective immunization efficacy of leukemia cell vaccines in BALB/c mice using BM185 cells transfected with B7.1/GM-CSF or B7.1/IL-2 encoding MIDGE vectors. Furthermore, we evaluated the protective effect of immunostimulatory molecules containing CpG sequence motifs (dSLIM-4S1).

Fig. 1 Schematic diagram of the (A) MIDGE vector<sup>α</sup> (16) and of the (B) MFG-based retroviral vector as shown (24). CMV, CMV promoter; LTR, long terminal repeat; pA, polyadenylation site.

**MATERIALS AND METHODS**

**Mice.** Eight-week-old female BALB/c mice were purchased from Harlan and Winkelmann (Borchen, Germany) and maintained under standard conditions. Experiments were performed with at least 5 mice in each group. All of the experiments were reviewed and approved by the Institutional Review Board and by the Animal Care Committee of the Senate of Berlin, Germany. Tumor growth of mice was monitored every second day by measuring two perpendicular diameters of the tumor. When the tumors reached a diameter >20 mm, mice were sacrificed. To detect systemic leukemia, peripheral blood (100 μl) from each mouse was collected from the tail vein. Heparinized blood was smeared on glass slides, dried, and stained with the Diff-Quick system (Baxter Dade AG, Düdingen, Switzerland). After fixation (fast green in methanol) blood slides were stained with eosin G and in thiazine dye in phosphate buffer (pH 6.6), and examined for leukemia cells.

**Cell Lines.** The wild-type-BM185 cell line, and the GM-CSF, IL-2, and B7.1 transduced BM185 cell lines were generated as described previously (10). In brief, the BM185 cell line was obtained from bone marrow cells of BALB/c mice transduced with a retroviral vector encoding the human M<sub>f</sub> 185,000 BCR-ABL fusion protein. IL-3 independent cells were grown in RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 100 units/liter penicillin/streptomycin, and 10<sup>−5</sup> M 2-mercaptoethanol, and clones were selected by limiting dilution.

**Expression Vectors.** The MIDGE gene expression system was designed to contain only the essential elements, as there is a CMV promoter, the gene of interest, and a termination site, hereby avoiding bacterial promoters and sequences coding for antibiotic selection markers (Fig. 1A). As genes of interest, the murine cDNA of GM-CSF, IL-2, or B7.1 were used. Generation of the MIDGE vector system was performed as described previously (16). Briefly, each coding sequence of interest was inserted into the plasmid vector downstream of a CMV promoter between the restriction sites KpnI and SacI. ODNs con-

---

<sup>α</sup> Internet address: http://www.midge.com.
Fig. 2 Structure (A) and sequence (B) of the dSLIM-4S1 ODN. The noncoding linear DNA construct contains unmethylated immunostimulatory CpG sequence motifs (depicted in bold, arrows) and is stabilized by single stranded thymidine loops.

consisting of a self-complementary sequence and separated by four thymidine bases ($T_d$) were synthesized by TIB MolBiol (Berlin, Germany). Annealing of the self-complementary ODN sequence results in the formation of a double-stranded stem with an EcoRI overlap on one side and a $T_d$ loop on the other side. The expression cassette was excised from the pG plasmid by an EcoRI digest. ODNs were added to the resulting solution and incubated with T4-DNA ligase to stabilize the linear vector against exonuclease activity. Vector backbone remnants were digested by HindIII and T7-polymerase. The final construct was separated from shorter ODN ligation products and nucleosides by anion-exchange high-performance liquid chromatography. The retroviral vectors were MFG-based with the long terminal repeat of the Moloney murine leukemia virus as described previously (Refs. 24, 25; Fig. 1B).

**Immunomodulatory Molecules.** Single-stranded hairpin 5'-phosphorylated ODN of the sequence 5'-GGTTCT-TCCGGGGCCTCTTTTTAAGAAGCCCC-3' and 5'-GAAGACGTTCCCAATGTTCATGTGAAAAC-3' were ligated with T4-DNA ligase (MBI Fermentas, Vilnius, Lithuania). After digestion with T7-polymerase and chromatographic purification, the DNA was ethanol precipitated and resuspended in PBS. The content of endotoxins was determined by an endotoxicity assay using a FACSCalibur (Becton Dickinson, San Diego, CA) and mouse anti-CD80 antibodies (PharMingen, Hamburg, Germany) 24 h after transfection of BM185 cells via electroporation or ballistic gene transfer or after retroviral transduction.

**Transfection.** Gene transfer via electroporation was performed as described previously (27). Briefly, 5 × 10⁶ BM185 cells were harvested at logarithmic phase and electroporated in a 0.4-cm cuvette (Eurogentec) at 300 V and 1050 µF using the Easyjet apparatus (Eurogentec, Seraing, Belgium). We used a total amount of 30 µg MIDGE vector per electroporation (for transfection with a single vector: 30 µg; for transfection with two vectors: 15 µg of each vector). The ballistic gene transfer was performed as described (28). Before transfection, 1 × 10⁸ BM185 cells were seeded on polylysine coated cell culture plates. After removal of the supernatant, we transfected BM185 cells using the Ballistic PDS-1000/He System (BIO-RAD Laboratories, Hercules, CA). The helium pressure (2200 p.s.i.) and vacuum circuits in the system accelerated 7 mg of 0.8–1.6 µm sized AU-165 gold microcarriers (ABCR, Karlsruhe, Germany) coated with a total amount 14 µg of MIDGE vector (for transfection with a single vector: 14 µg; for transfection with two vectors: 7 µg of each vector) into the BM185 target cells. Retroviral gene transfer was performed as described (25). Briefly, stable packaging lines (ψ crip) were used to produce high titers of retroviral vectors. BM185 cells growing in logarithmic phase were washed with fresh medium, mixed in the retroviral supernatants plus protamine sulfate (6 µg/ml), subjected to spinoculation for 2 h at 1000 × g and 32°C, and incubated for 16 h at 32°C and 5% CO₂. Double-positive cell clones (B7.1/GM-CSF and B7.1/IL-2, respectively) were generated via sequential transduction. After B7.1 transduction and selection of B7.1-positive clones, the clones were transduced with GM-CSF or IL-2 and selected for transgene expression. Transduced clones that showed persistent transgene expression were selected and served as positive controls for BM185 cells transfected with nonviral techniques.

**Expression Analysis.** GM-CSF expression was analyzed by ELISA (R&D Systems, Wiesbaden, Germany) with an ELISA reader (SLT Labinstruments, Grödig, Austria) 24 h after transfection. B7.1 expression was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, San Diego, CA) and mouse anti-CD80 antibodies (PharMingen, Hamburg, Germany) 24 h after transfection of BM185 cells via electroporation or ballistic gene transfer or after retroviral transduction.

**Immunization of Mice.** Double transfections of BM185 cells were performed via electroporation, or ballistic or retroviral gene transfer as described above. As control, vaccine nontransfected BM185 cells were used. After irradiation at 20 Gy, using a ¹³₇Cs γ radiation (OB29/4, STS, Braunschweig, Germany), cells were washed three times, resuspended in Hank’s buffered saline with heparin (20 units/ml) at 1 × 10⁷ cells/ml and kept on ice until injection. Mice were injected s.c. on days −21 and −7, and received 2 × 10⁶ irradiated vaccine cells on each day in a volume of 200 µl and in addition, as indicated, 50 µg of dSLIM-4S1 as immunomodulator into the shaved left-side inguinal region. The time interval between transfection and immunization was 24–36 h, reflecting a peak in transgene expression and no time-dependent decay at this point.

**Leukemia Challenge of Mice.** BM185 cells and transduced/transfected BM185 cells were cultured in RPMI 1640, supplemented with 5% FCS, 2 mM l-glutamine, 100 units/liter penicillin/streptomycin, and 10⁻⁵ M 2-mercaptoethanol. For leukemia challenge, BM185 cells in exponential growth were harvested, washed three times, resuspended in Hank’s buffered saline with heparin (20 units/ml) at 1 × 10⁶ cells/ml, and kept on ice until injection. Mice were anesthetized by i.m. injection of 200 µl of a mixture containing 250 µl of xylazine hydrochloride (2.85 mg/ml) and ketamine hydrochloride (42.8 mg/ml), and 750 µl NaCl (0.9%). Mice received s.c. injections of 1 × 10³ BM185 (LD₅₀ = 100 cells) cells in a volume of 100 µl into the shaved right-side gluteal region on day 0. This leukemia...
challenge dose has been shown to induce a tumor size of 20-mm diameter within 3 weeks in untreated mice.

**Statistical Analysis.** Expression analysis was performed in six separate transfection experiments. Differences of transfection efficacy and viability of transfected BM185 cells were analyzed by using the unpaired t test. Vaccination experiments were performed with at least 5 mice in each group. The end point of each experiment was reached either when the induced tumors reached a diameter >20 mm (criteria for sacrifice) or when mice had a tumor-free survival up to 100 days after leukemia challenge. Because mice did not develop tumors after 100 days postleukemia challenge, life span of surviving mice was arbitrarily set to 100 days. Parameters of immunization efficacy were tumor growth, time of tumor-free and overall survival, and number of surviving mice. Statistical analysis for comparison of tumor-free and overall survival was performed by using the unpaired t test.

**RESULTS**

**Comparison of Electroporation, and Retroviral and Ballistic Gene Transfer in BM185 Cells.** To evaluate the efficiency of nonviral gene transfer techniques regarding their potential use for the generation of leukemia cell vaccines in clinical trials, we transfected murine BM185 cells, which do not express B7.1 or GM-CSF, with MIDGE vectors encoding murine B7.1 or GM-CSF by ballistic gene transfer or electroporation. Retrovirally transduced BM185 cell lines served as controls. We determined the transfection rate 24 h after transfection by analyzing the expression of B7.1 molecules on the cell surface using flow cytometry and the amount of secreted GM-CSF using ELISA. BM185 cells transfected with B7.1 showed a significant lower transfection rate after ballistic gene transfer (mean 30.9%) than after electroporation (mean 82.6%; \( P < 0.0001 \)) and retroviral transduction (mean 96.4%; \( P < 0.0001 \); Fig. 3A). In contrast, after gene transfer of GM-CSF, the highest secreted GM-CSF level (per \( 1 \times 10^6 \) cells) was found after ballistic transfer (mean 614 pg), which was significantly higher than after electroporation (mean 228 pg; \( P < 0.0001 \)) and comparable with the GM-CSF level after retroviral transduction (mean 525 pg; Fig. 3B). The viability of B7.1-transfected BM185 cells after ballistic gene transfer was higher (mean 61.0%) than after electroporation (mean 49.1%), although the difference was not significant (\( P = 0.054 \); Fig. 3C). These data suggest that electroporation leads to a higher number of transfected cells (as detected via B7.1 expression), whereas ballistic transfer yields a higher overall expression (as detected via GM-CSF secretion).

**Optimization of Immunization Controls.** The application of only 1000 BM185 cells induces a pre-B-ALL in mice, which die 3 weeks after i.v. injection or develop a tumor >20 mm in diameter after s.c. injection (10). Thus, we compared different doses of BM185 leukemia cells for challenge. Surprisingly, even a challenge with as low as 500 BM185 cells injected s.c. induced tumors >20 mm in diameter in all of the mice after a mean time of 19.9 days (data not shown), which underlines the aggressiveness of our leukemia challenge model. Then we compared the outcome of mice preimmunized with nontransfected, irradiated BM185 cells and of mice that received no vaccine. Although BM185-vaccinated mice showed a longer mean tumor-free survival (21.2 days versus 9.5 days) and overall survival (30.0 days versus 19.9 days) than nonvaccinated mice, there was no significant difference of tumor growth rate (data not shown), and only 1 of 10 mice survived in the BM185-vaccinated group for unknown reasons (Table 1).

**Immunization of Mice with B7.1/IL-2 Cell Vaccines.** We analyzed if and to what extent vaccination with genetically modified leukemia cells can protect mice against a lethal leukemia challenge. We first evaluated BM185 cells electroporated or retrovirally transduced with B7.1 and IL-2 for their use as cell vaccines. Vaccination before the leukemia challenge resulted in a reduced tumor growth rate for both groups (Fig. 4B). In addition, mean tumor-free survival (37.6 days and 48.8 days versus 9.5 days), overall survival (54.0 days and 57.6 days versus 19.9 days), and survival rate (20% and 40% versus 0%) were increased in mice that received the B7.1- and IL-2-expressing vaccines generated by electroporation or retroviral gene transfer compared with the no vaccine group (Fig. 4B; Table 1). All of the surviving mice were tumor-free. In contrast, therapeutic vaccination with B7.1/IL-2 expressing cells 3 days after the leukemia challenge did not lead to prolonged survival, but blast counts in peripheral blood were decreased in those mice that received B7.1/IL-2-expressing vaccines (BM185 versus electroporated B7.1/IL-2 vaccine versus retroviral B7.1/IL-2 vaccine: 15.8% versus 8.0% versus 4.0% blasts; data not shown).

**Immunization of Mice with B7.1/GM-CSF Cell Vaccines.** We then tested B7.1- and GM-CSF-expressing BM185 cells, generated by retroviral gene transfer, electroporation, or ballistic gene transfer, as protective vaccines. The efficacy of the B7.1/GM-CSF vaccine generated by electroporation was higher in terms of mean tumor-free survival (47.2 days versus...
Cell-based Vaccination of Ph\(^+\) ALL

3146 Cell-based Vaccination of Ph\(^+\) ALL

Table 1 Survival after protective immunization with leukemia cell vaccines\(^a\)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Gene transfer</th>
<th>Tumor-free survival (d)</th>
<th>Overall survival (d)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No vaccine</td>
<td>—</td>
<td>9.5</td>
<td>19.9</td>
<td>0.0</td>
</tr>
<tr>
<td>BM185</td>
<td>—</td>
<td>21.2</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>B7.1/GM-CSF</td>
<td>B</td>
<td>29.6</td>
<td>44.4</td>
<td>20.0</td>
</tr>
<tr>
<td>B7.1/IL-2</td>
<td>E</td>
<td>37.6</td>
<td>54.0</td>
<td>20.0</td>
</tr>
<tr>
<td>B7.1/IL-2</td>
<td>R</td>
<td>48.8</td>
<td>57.6</td>
<td>40.0</td>
</tr>
<tr>
<td>B7.1/GM-CSF</td>
<td>E</td>
<td>47.2</td>
<td>54.2</td>
<td>40.0</td>
</tr>
<tr>
<td>B7.1/GM-CSF/dSLIM</td>
<td>E</td>
<td>64.4</td>
<td>70.8</td>
<td>60.0</td>
</tr>
<tr>
<td>B7.1/GM-CSF</td>
<td>R</td>
<td>68.8</td>
<td>92.0</td>
<td>80.0</td>
</tr>
</tbody>
</table>

\(^a\) Genetically modified leukemia cell vaccines were compared with the no vaccine control group.

DISCUSSION

For clinical application of immunotherapy with genetically modified leukemia cells, there is a need to achieve a balance between high efficacy of gene transfer and survival of primary human ALL blasts, which are fragile and poorly tolerant to extended culture (29). Leukemia cells used as vaccines have to be irradiated to prevent proliferation in vivo and, therefore, will only express the transgenes for a few days. Thus, gene transfer methods that lead to stable gene expression may not be superior in terms of vaccine efficacy compared with methods that result in transient gene expression. Dilloo et al. (30) described the high-efficiency transduction of normal and malignant human hematopoietic cells by a novel herpes simplex vector that shows transient expression. One limitation of this vector is the induction of an antiherb simplex response, which is similar to phenomena observed with adenoviral vectors. Alternatively, primary ALL cells can be transduced by lentiviral vectors (31). However, transduction efficacy varies considerably and, although lentiviral vectors have thus far been shown to be free of replication-competent virus, this vector has not been used in clinical trials because of safety concerns. At present, retroviral vectors are the most widely used for the transfer of genes to hematopoietic cells.

vaccination with B7.1/GM-CSF-expressing BM185 cells resulted in no significant effect on survival rates, but blast counts were moderately effected by the vaccines (BM185 versus electroporated B7.1/GM-CSF vaccine versus retroviral B7.1/GM-CSF vaccine: 15.8% versus 9.8% versus 4.8% blasts; data not shown).

Administration of Immunomodulatory dSLIM-4S1 as Adjuvants. To improve the B7.1/GM-CSF vaccine generated by electroporation, we designed a noncoding dSLIM-4S1 containing three nonmethylated CpG motifs (Fig. 2) for use as an immunomodulator. Additional application of dSLIM-4S1 to the electroporated B7.1/GM-CSF vaccine resulted in a longer mean tumor-free (64.4 days versus 47.2 days) and overall survival (70.8 days versus 54.2 days), and a higher survival rate (60% versus 40%) compared with the B7.1/GM-CSF vaccine alone. All of the surviving mice were disease-free and showed no tumor growth (Table 1). Thus, the combination of the B7.1/GM-CSF vaccine and the immunomodulator dSLIM-4S1 was almost as effective as the B7.1/GM-CSF vaccine generated by retroviral gene transfer.

Fig. 4 Immunization of mice with B7.1- and IL-2-transfected BM185 vaccines. Depicted is survival of mice (A) and tumor growth (B), measured every second day. Before the leukemia challenge on day 0, mice received either no vaccine (cross) or were vaccinated on days -21 and -7 with irradiated IL-2- and B7.1-expressing BM-185 cells, generated by electroporation (●) or by retroviral gene transfer (○).

29.6 days), overall survival (54.2 days versus 44.4 days), and survival rate (40% versus 20%) compared with the same vaccine generated by ballistic gene transfer. As expected, the B7.1/GM-CSF vaccine generated by retroviral gene transfer was even more effective than the vaccine generated by electroporation, leading to a decreased tumor growth rate (Fig. 5B), a prolonged mean tumor-free and overall survival (68.8 days and 92.0 days, respectively), and a survival rate of 80% (Fig. 5A; Table 1). In general, all of the surviving mice never developed a tumor. Regardless of whether generated by retroviral gene transfer or electroporation, the B7.1/GM-CSF-expressing vaccines were superior to the B7.1/IL-2-expressing vaccines (Table 1). In keeping with therapeutic B7.1/IL-2 vaccination, a therapeutic
of cells were transfected after electroporation (Fig. 3A). In contrast, after ballistic gene transfer of GM-CSF transfected cells produce more than twice the amount of GM-CSF compared with the electroporated cells. This can be explained by the fact that less BM185 cells were transfected by ballistic gene transfer but transfected cells carry more copies of expression vectors compared with electroporated cells. Indeed, after ballistic gene transfer up to 30,000 plasmid copies have been found in transfected K562 leukemia cells.\(^4\) In view of the better protection of mice that received cell vaccines generated by electroporation compared with mice that received cell vaccines generated by ballistic gene transfer, it seems likely that moderate expression of cytokines or costimulatory molecules by a higher number of vaccine cells might be superior in terms of vaccine efficacy than a high expression by a few vaccine cells.

The role of the costimulatory molecule B7.1 (8, 34–36), the function of IL-2 (37, 38), and the role of GM-CSF (6, 39–44) in mediating immune responses to solid or hematological malignancies have been extensively studied by several authors in preclinical and clinical studies with autologous tumor vaccines. In this study we evaluated the protective immunization with BM185 leukemia cell vaccines expressing B7.1/GM-CSF or B7.1/IL-2 in a murine leukemia challenge model. Because this system is based on a proliferating cell line, which represents a good target for retroviral transduction, we did not expect the nonviral methods to be superior in terms of transfection and immunization efficacy. But both gene transfer and vaccination experiments demonstrate that nonviral techniques can be optimized to protect against a highly aggressive leukemia, which is lethal to 100% of nonvaccinated control mice. In general, mice that received the B7.1/GM-CSF vaccine were better protected than mice that received the B7.1/IL-2 vaccine. The coexpression of B7.1 and GM-CSF was also significantly more effective at promoting leukemia rejection than the expression of B7.1 and IL-2 when BM185 cells were transduced by retroviral gene transfer as published previously (25). The protection of mice was T-cell dependent, because the vaccine did not modify leukemia development in immunodeficient mice. Furthermore, it was shown that vaccination with irradiated BM185 cells coexpressing B7.1 and GM-CSF stimulated the highest CTL activity (25). The lower efficacy of the B7.1/IL-2 vaccine may be explained that T-cell anergy is not present in our leukemia model and, therefore, bypassing helper T function by IL-2 synthesis (7) may not be necessary for the induction of CTL.

Presumably because of evolutionary pressure, nonmethylated CpG motifs are far more frequently present in the genomes of bacteria and viruses than of vertebrates (45). Recently, Zimmermann et al. (46) showed that even 15–20 days after an inoculation with a lethal dose of leishmanium major, mice could be cured by the application of three consecutive doses of CpG oligonucleotides, which induced a Th1-mediated immune response. We observed here that coadministration of dSLIM-4S1, containing three nonmethylated CpG sites, moderately enhanced the protection of the cell-based vaccine. Mice that received the B7.1/GM-CSF vaccine generated by electroporation in combination with the CpG oligonucleotide dSLIM-4S1 showed a

![Image](63x330 to 291x699)

**Fig. 5** Immunization of mice with B7.1 and GM-CSF cotransfected BM185 vaccines. Depicted is survival of mice (A) and tumor growth (B), measured every second day. Before the leukemia challenge on day 0, mice received either no vaccine (cross) or were vaccinated on days −21 and −7 with irradiated GM-CSF- and B7.1-expressing BM-185 cells, generated by electroporation (gray circles) or by retroviral gene transfer (○). Additional application of dSLIM-4S1 molecules to the vaccines generated by electroporation is shown in ●.

Because our experimental model allows both cell cycle-dependent and -independent gene transfer, we first compared nonviral gene transfer methods such as electroporation and ballistic gene transfer with retroviral gene transfer in BM185 cells. Whereas viability of transfected BM185 cells was moderately higher after ballistic gene transfer than after electroporation, only ~30% of BM185 cells express the membrane-bound ligand B7.1 after ballistic gene transfer, whereas >80% of cells were transfected after electroporation (Fig. 3A). In

\(^4\) B. Wittig, unpublished observations.
longer overall time of survival than the control and was as effective as the retroviral generated B7.1/GM-CSF vaccine. Although we did not investigate the specific activation of immune cells by dSLIM molecules in this study in detail, we have extensively analyzed the effect of covalently closed CpG-containing dSLIM molecules on peripheral blood mononuclear cells from human donors previously. Whereas CpG-containing dSLIM molecules induced the secretion of IL-2, IFN-γ, IL-12p40, and IL-6, the same molecules lacking CpG motifs did not induce cytokine secretion. The induction of TH1-type cytokine production and thereby activation of various immune cells by CpG-containing oligonucleotides was also described previously (19, 22). This unspecific accelerated immune status can be designated as signal "0," which is needed for a specific immune response via antigen-presentation and costimulation signals (47, 48). Thus, we suggest that the immunostimulatory effect of CpG oligonucleotides can be directed not only against bacterial or viral infections but also against hematologic malignancies in the presence of tumor-associated antigens expressed by the vaccinated BM185 cells.

Although systemic leukemia is more closely resembled by intravenous application of leukemia cells, we favored the s.c. injection mode, because local leukemia growth can be better quantified than leukemia development after i.v. injections. Tumor growth was monitored in this study every second day. Furthermore, blast counts in peripheral blood indicated that leukemia had spread also systemically and was not confined to the s.c. injection site. Local tumor growth might be more sensitive to immunotherapy than system growth, but we have shown previously that preimmunized mice were protected also when BM185 cells were injected i.v. (25).

In conclusion, these experiments showed that vaccination with genetically modified leukemia cells generated by nonviral gene transfer and a novel minimalistic linear vector system (MIDGE) can protect mice against a syngeneic leukemia challenge. Secondly, leukemia cell vaccines generated by electroporation were more effective than those generated by ballistic transfer. Thirdly, the coexpression of B7.1 and GM-CSF lead to a better immunization compared with the coexpression of B7.1 and IL-2. Finally, the coadministration of a newly developed DNA construct dSLIM-4S1 containing unmethylated CpG motifs enhanced the efficacy of the B7.1/GM-CSF cell vaccine, which is similar to the retrovirally generated B7.1/GM-CSF cell vaccine, but avoiding the risks attached to the retroviral gene transfer system. A prerequisite for clinical leukemia cell vaccination trials is the extensive analysis of transfection efficacy in human primary leukemia cells. Nevertheless, we suggest that cell-based vaccines should be considered for patients with Ph+ ALL during remission to prevent an ALL relapse.

ACKNOWLEDGMENTS

We thank L. Badiali and M. Löwe for excellent technical help.

REFERENCES


17. Boretti, F. S., Leutenegger, C. M., Mislin, C., Hofmann-Lehmann, R., Konig, S., Schoeff, M., Junghans, C., Fehr, D., Huettn, S. W.,

Habel, A., Flynn, J. N., Aubert, A., Pedersen, N. C., Wittig, B., and Lutz, H. Protection against HIV challenge infection by genetic vaccina-


Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G. B., Rocken, M., Wagner, H., and Heeg, K. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmani-


Protection of Mice against Philadelphia Chromosome-positive Acute Lymphoblastic Leukemia by Cell-based Vaccination Using Nonviral, Minimalistic Expression Vectors and Immunomodulatory Oligonucleotides

Joachim Köchling, Sven A. König-Merediz, Renata Strippecke, et al.


Updated version
Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/9/8/3142

Cited articles
This article cites 46 articles, 21 of which you can access for free at: http://clincancerres.aacrjournals.org/content/9/8/3142.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/9/8/3142.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.