Immunization of Melanoma Patients with BEC2-Keyhole Limpet Hemocyanin plus BCG Intradermally Followed by Intravenous Booster Immunizations with BEC2 to Induce Anti-GD3 Ganglioside Antibodies

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ABSTRACT

BEC2 is an anti-idiotypic mouse monoclonal antibody that mimics GD3 ganglioside. Previous clinical trials demonstrated that intradermal immunization using 2.5 mg of BEC2 with BCG or i.v. immunization with 10 mg of BEC2 can induce anti-GD3 antibodies in a subset of patients. We hypothesized that combining these two immunization strategies might be more effective in inducing anti-GD3 antibodies and that conjugation of BEC2 to keyhole limpet hemocyanin (KLH) would further enhance the immunogenicity of BEC2. In this clinical trial, 18 melanoma patients who were free of disease after complete surgical resection within 1–6 months received intradermal immunizations on weeks 0, 2, 4, 6, and 10 with 2.5 mg of BEC2 conjugated to KLH and mixed with BCG (BEC2-KLH/BCG). Booster immunizations of 10 mg of unconjugated BEC2 were administered i.v. on weeks 24, 37, and 50. Four of 18 patients (22%) developed IgM anti-GD3 antibodies. No IgG anti-GD3 antibodies were detected. All four responding patients developed anti-GD3 IgM during immunization with BEC2-KLH/BCG; only one patient demonstrated a boost of the IgM anti-GD3 titer during the i.v. immunizations. Thirteen of the patients are free of melanoma (3 after undergoing re-resection for local relapse); 14 patients (78%) remain alive with a median follow-up of 28 months. These results confirm our previous trial, showing that BEC2 with BCG can induce anti-GD3 antibodies in patients. The data do not provide evidence that conjugation to KLH increases the immunogenicity of BEC2 when it is administered with BCG.

INTRODUCTION

GD3 ganglioside is a glycolipid that is abundantly expressed on melanoma but is expressed only to a limited extent on normal tissues. Infusion of MAbs against GD3 has resulted in clinical responses in metastatic melanoma patients (1), indicating that GD3 can be an important target for immunotherapy. With the goal of inducing active immunity against GD3, we developed an anti-idiotypic Mab vaccine, designated BEC2, that mimics GD3 and can induce anti-GD3 antibodies in animals (2). A series of clinical trials carried out in melanoma patients who were free of disease after complete surgical resection demonstrated that anti-GD3 antibodies could be induced in patients using one of two BEC2 formulations. BEC2 (2.5 mg) mixed with BCG injected i.d. induced anti-GD3 antibodies (3), as did i.v. administration of BEC2 at doses of ≥10 mg (4). Each of these approaches induced detectable anti-GD3 antibodies in 20–25% of patients immunized.

We carried out a clinical trial incorporating what had been learned in the previous BEC2 trials in an attempt to increase the percentage of patients developing detectable anti-GD3 antibodies after immunization. We reasoned that an initial immunization with BEC2 plus BCG followed by i.v. booster immunizations with BEC2 might result in a higher incidence of anti-GD3 antibody induction. We were also aware that previous trials with anti-idiotypic MAb (5) and ganglioside vaccines (6) demonstrated that conjugation to KLH can increase vaccine immunogenicity. Therefore, in this clinical trial, patients were initially immunized i.d. with BEC2 conjugated to KLH mixed with BCG (BEC2-KLH/BCG) at the BEC2 dose and schedule used previously. Subsequently, patients received i.v. booster immunizations with unconjugated BEC2 alone at a dose of 10 mg. The primary end point of the trial was the induction of anti-GD3 antibodies.

MATERIALS AND METHODS

Patient Selection. Patients with American Joint Committee on Cancer stage II (>4 mm deep), stage III, or stage IV melanoma who had undergone complete surgical resection in the previous 1–6 months were eligible. Stage IV patients were not eligible if their melanoma had been metastatic to the central nervous system. Patients were required to have a normal WBC count and differential. Patients were excluded if they: (a) had received antimelanoma therapy within the past 4 weeks (6

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3 The abbreviations used are: MAb, monoclonal antibody; i.d., intradermal(ly); KLH, keyhole limpet hemocyanin; CFU, colony-forming units.
weeks for nitrosoureas); (b) had a history of another invasive malignancy within the past 5 years; (c) had other serious illnesses or known immunodeficiencies; (d) required systemic antihistamines, nonsteroidal anti-inflammatory drugs, or steroids; or (e) had undergone splenectomy or radiotherapy to the spleen. Pregnant and breastfeeding women were excluded, and women of childbearing potential were required to use adequate methods to avoid pregnancy. All patients signed written informed consent.

**Vaccine Formulations.** BEC2 was purified from virus-free lipoprotein-free cell culture supernatants by Celltech (Slough, England), using Protein A affinity chromatography and anion-exchange chromatography. The final product was >95% pure, as determined by SDS-PAGE under reducing conditions. BEC2 was supplied by ImClone Systems (Somerville, NJ) in sterile PBS at a concentration of 2.5 mg/ml in single-dose vials. BEC2 was conjugated to KLH (Akzo Organon Teknika, Rockville, MD) by glutaraldehyde conjugation. To do this, BEC2 was mixed with KLH (10:1, w/w) in a sterile, endotoxin-free conical tube. While the solution was gently vortexed, we added 1% glutaraldehyde drop by drop to a final glutaraldehyde concentration of 0.05%. The mixture was further vortexed for 5 min and then allowed to incubate at room temperature for an additional 55 min without vortexing. The reaction was stopped by the addition of 3 M glycine (pH 7.0) and dialysis against PBS (pH 7.2–7.4) to remove unreacted glutaraldehyde and excess glycine. Each batch was tested for adequacy of conjugation by SDS-PAGE under nonreducing conditions and size-exclusion chromatography. To confirm that BEC2 immunoreactivity was retained after conjugation to KLH, the BEC2-KLH conjugate was tested for its ability to bind R24, the MAb against which BEC2 was conjugated to KLH, the BEC2-KLH conjugate. The final product was freeze-dried from a sterile PBS solution at a concentration of 2.5 mg/ml in single-dose vials. The BEC2-KLH conjugate was stored at −80°C for serological analysis.

**Immunization with BEC2-KLH/BCG.** The starting dose of BCG was 3 × 10^6 CFU per immunization. In patients who had a history of tuberculosis or a positive skin reaction to PPD or who had previously received BCG vaccine, the starting dose of BCG was decreased by 33% (3 × 10^5, 1 × 10^6, 3 × 10^5, and 1 × 10^5 CFU per immunization). In patients who had a history of tuberculosis or a positive skin reaction to PPD or who had previously received BCG vaccine, the starting dose of BCG was decreased by 33% (3 × 10^5, 1 × 10^6, 3 × 10^5, and 1 × 10^5 CFU per immunization). In patients who had a history of tuberculosis or a positive skin reaction to PPD or who had previously received BCG vaccine, the starting dose of BCG was decreased by 33% (3 × 10^5, 1 × 10^6, 3 × 10^5, and 1 × 10^5 CFU per immunization). In patients who had a history of tuberculosis or a positive skin reaction to PPD or who had previously received BCG vaccine, the starting dose of BCG was decreased by 33% (3 × 10^5, 1 × 10^6, 3 × 10^5, and 1 × 10^5 CFU per immunization). In patients who had a history of tuberculosis or a positive skin reaction to PPD or who had previously received BCG vaccine, the starting dose of BCG was decreased by 33% (3 × 10^5, 1 × 10^6, 3 × 10^5, and 1 × 10^5 CFU per immunization).

Serum was collected pretreatment and on weeks 2, 4, 6, 8, 10, and 12 and cryopreserved at −20°C for serological analysis.

**Booster Immunizations with i.v. BEC2.** Booster immunizations, consisting of 10 mg of unconjugated BEC2 infused i.v., were administered on weeks 24, 37, and 50. Serum was collected for serological analyses at the time of each booster immunization and 2 weeks afterward.

**Serological Analyses.** Serum was tested for the presence of anti-BEC2 IgG and anti-GD3 IgG and IgM by ELISA against purified GD3 as described previously (3, 7). Internal standards using either human IgM and IgG MAb against GD3 (for the anti-GD3 assays) or a reference serum sample (for the anti-BEC2 assays) were run with each assay to ensure adequate and reproducible sensitivity. The anti-BEC2 ELISA detected anti-mouse IgG and was not specific for antibodies against BEC2 idiotopes. A patient was considered to have developed anti-GD3 antibodies if at least two posttreatment sera samples reproducibly demonstrated a ≥4-fold increase in anti-GD3 titer (cutoff, A_{405 nm} = 0.05) compared to the pretreatment sample. This strict criterion was established based on our previous serological studies in patients immunized with BCG alone and avoids confusion with transient, low-level, nonspecific reactivity that can be induced by BCG and other adjuvants. For anti-BEC2 IgG titers, we used a cutoff of A_{405 nm} = 0.1.

**RESULTS**

**Patient Characteristics.** Eighteen patients (11 men and 7 women) were accrued to the trial (Table 1). The median age was 48.5 years. Eleven of the patients (61%) had axial primary sites, 6 (33%) had primaries on the extremities, and 1 patient had an unknown primary. One patient had American Joint Committee on Cancer stage II melanoma, 16 patients had stage III melanoma, and 1 patient had stage IV melanoma. Among the stage III patients, 10 of 16 had only a single lymph node or in-transit metastasis, 2 patients had more than four involved.
lymph nodes, and the remaining 4 patients had between two and four involved lymph nodes and/or in-transit metastases. The median time from complete surgical resection to the first vaccination with BEC2 was 3.7 months (range, 1.2–5.7 months).

All 18 patients received the five i.d. immunizations with BEC2-KLH/BCG. Thirteen patients completed the entire vaccination schedule. Four patients were removed from the study because of recurrence before receiving the first i.v. booster immunization; one patient received a single i.v. booster immunization before being removed from the study because of recurrent melanoma.

Serological Responses. Of the 18 patients, 4 (22%; exact 95% confidence interval, 6–48%) developed IgM anti-GD3 antibodies detectable by ELISA (Fig. 1); no patient developed detectable IgG antibodies. In all four patients, the anti-GD3 IgM antibodies were induced as a result of immunization with BEC2-KLH/BCG and were transient, lasting between 2 and 4 weeks. In one case (patient 3; Fig. 1A), subsequent immunizations with i.v. BEC2 resulted in a repeat boost in IgM anti-GD3 titers. We did not observe an IgG anti-GD3 amnestic response in any of the patients. Anti-GD3 antibodies were not detectable by immuno-TLC (8) or by flow cytometry against GD3+ melanoma cell lines, suggesting that these anti-GD3 antibodies had a relatively low avidity for cell surface GD3.

Consistent with our previous observation that the addition of BCG as an immune adjuvant resulted in 100% of patients developing anti-BEC2 IgG (3, 9), all patients developed IgG antibodies against BEC2. However, it appeared that conjugation to KLH had resulted in anti-BEC2 titers that were lower than the titers we had observed previously in melanoma patients immunized with unconjugated BEC2 mixed with BCG (3). To test this, we analyzed the anti-BEC2 IgG titers 2 weeks after the fifth immunization in our current patients immunized with BEC2-KLH/BCG and compared them with sera from the same time point from melanoma patients immunized with BEC2/BCG in our previous trial (3). The IgG titers of patients immunized with BEC2-KLH/BCG (median titer, 1:12,800; range, 1:400–1:409,600) were significantly lower than those of patients immunized with BEC2/BCG (median titer, 1:101,200; range, 1:12,800–1:406,600) by Wilcoxon rank-sum test ($P = 0.01$).

One patient (patient 4) was found to be pregnant by urine human chorionic gonadotropin-β determination, 2 weeks after receiving her third and final i.v. booster immunization. She went on to deliver a full-term healthy girl and voluntarily provided cord blood and colostrum for serological analysis. Anti-GD3 antibodies had not been detected in this patient’s serum, and so we were not surprised that there were no detectable anti-GD3 antibodies in cord blood or colostrum. IgG anti-BEC2 antibodies were detectable in the cord blood although neither IgG nor IgA anti-BEC2 antibodies were detected in the colostrum. Fig. 2 shows that anti-BEC2 IgG were induced by immunization with BEC2-KLH/BCG and further boosted to a peak titer of 1:204,800 after the second i.v. booster immunization. The final i.v. booster immunization was administered on week 50, and 2 weeks later, the IgG anti-BEC2 titer was 1:25,600. Serum from peripheral blood was next sampled on week 92 (40 weeks later), at which time the titer had decreased only by two tube dilutions (1:6400). Cord blood, sampled on week 85 (35 weeks after the last immunization and 7 weeks before the week 92 serum),

![Fig. 1](clincancerres.aacrjournals.org) IgM anti-GD3 antibody responses in four immunized patients. A, patient 3; B, patient 9; C, patient 12; D, patient 14. Y axis, IgM anti-GD3 antibody titers (cutoff $A_{405} = 0.05$); arrows, immunizations. The first five arrows in each plot indicate i.d. immunization with BEC2-KLH/BCG; remaining arrows indicate i.v. BEC2 immunizations. Patient 12 (C) received only the first five immunizations (i.d. immunization with BEC2-KLH/BCG) before being taken off study due to progression of melanoma.
showed an anti-BEC2 IgG titer of 1:800. This showed that (a) the IgG anti-BEC2 antibodies were long-lived and (b) a proportion of these IgG antibodies could cross the placenta resulting in detectable titers in the cord blood. There have been no apparent effects of these antibodies on the normal development of the baby.

**Toxicity.** The vaccinations were well tolerated. The only grade III toxicity was skin toxicity, which was seen in all 18 patients at the site of i.d. immunizations and was due to BCG. Seven patients had fever (four had grade I; three had grade II) and three patients had transient fatigue (two had grade I; one had grade II).

**Disease Recurrence and Overall Survival.** Eight patients (44%) developed recurrent melanoma 3–14 months after the first vaccination. In three of those patients, the recurrences were local relapses that were resected, leaving the patients free of melanoma. One patient is alive with metastatic melanoma; the other four have died of metastatic melanoma. Ten patients (56%) are relapse free, with a median follow-up from resection of 31 months (range, 25–38 months).

Overall, 14 of 18 patients (78%) remain alive, with a median follow-up of 28 months after resection (range, 21–38 months). Fig. 3 represents a Kaplan-Meier plot of relapse-free and overall survival of the 18 patients immunized on this study.

**DISCUSSION**

The results of this clinical trial extend our experience in the use of BEC2 to immunize patients against GD3. We had previously found that i.d. immunization of melanoma and small cell lung cancer patients with BEC2+BCG resulted in induction of anti-GD3 antibodies in 8 of 29 patients (28%; 95% confidence intervals 13–47%; Refs. 3 and 9). We also found that i.v. immunization with BEC2 at doses of $\geq 10$ mg induced anti-GD3 antibodies in 3 of 16 (19%) melanoma patients (4). In an attempt to increase the percentage of immunized patients producing anti-GD3 antibodies, we attempted to combine these two vaccination approaches in this clinical trial. In addition, BEC2 was conjugated to KLH for the i.d. vaccinations in an attempt to enhance further the immunogenicity of the BEC2 molecule.

We found that this vaccination regimen induced anti-GD3 antibodies in a percentage of patients similar to that in our previous trials. In this study, 4 of 18 patients (22%) developed detectable IgM anti-GD3 antibodies. All four responding patients developed anti-GD3 antibodies as a result of immunization with BEC2-KLH/BCG; no patient developed anti-GD3 antibodies during the i.v. immunization period, and only one of the four responding patients demonstrated a repeat rise in IgM anti-GD3 titer as a result of the i.v. immunizations. These observations suggest that, when BCG is used as an adjuvant, conjugating BEC2 to KLH did not increase the immunogenicity compared to using unconjugated BEC2. These results are also consistent with our previous experience that the anti-GD3 antibody response induced by BEC2 can be self-limited over time and that additional immunizations often do not result in reboosting of the antibody response.

In analyzing the titers of anti-BEC2 IgG induced in this trial, we found evidence that conjugation to KLH reduced the immunogenicity of BEC2. This is consistent with the observation that no patient immunized in this study developed detectable IgG anti-GD3 antibodies, whereas in our previous trial using BEC2+BCG, we were able to detect the induction of IgG anti-GD3 antibodies (3). This implies that, despite conjugation to KLH, which would provide additional TH epitopes, the vaccination scheme did not provide sufficient signals to induce isotype switching of the anti-GD3 antibodies to IgG. It is possible that the process of conjugation, which nonspecifically

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4 P. B. Chapman, unpublished results.
cross-links proteins, sufficiently altered critical BEC2 epitopes and that this resulted in reduced immunogenicity.

During the course of this study, one patient, who did not develop anti-GD3 antibodies, became pregnant. She delivered a full-term, healthy baby girl and voluntarily provided a sample of cord blood and colostrum for immunological testing. We found that the cord blood contained anti-BEC2 IgG, although the titers were somewhat lower than in the peripheral serum measured 7 weeks later. There were no anti-BEC2 or anti-GD3 antibodies in the colostrum. This is the first report of which we are aware looking at the ability of human antimouse immunoglobulin antibodies to cross the placenta or to enter the colostrum. There was no evidence that human antimouse immunoglobulin antibodies—in this case, anti-BEC2 antibodies—interfered with normal embryogenesis.

Although 8 of 18 patients developed recurrences, in 3 of the patients, the recurrences were local and could be completely resected. Overall, 14 of 18 patients (78%) are alive at a median follow-up of 28 months; 13 of the 14 patients remain free of melanoma. This survival rate is similar to the 64% rate that we observed previously in melanoma patients immunized with unconjugated BEC2+BCG (3). Although this survival rate is higher than would be expected from historical controls (10–12), given the small sample size, the fact that this was a selected population, and the variability in 2-year survival rates among historical controls, we cannot make any firm conclusions regarding the effect of BEC2 immunization on survival in melanoma patients.

We note that, as in our previous trials with BEC2, relapse and survival did not strictly correlate with our ability to detect the induction of anti-GD3 antibodies. One possibility remains the insensitivity of our assay system. Another possibility is that immunological effector mechanisms other than anti-GD3 antibodies are induced by BEC2 immunization. Future studies will be directed toward testing this second hypothesis.

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