Phase I Trial of a Bivalent Gangliosides Vaccine in Combination with β-Glucan for High-Risk Neuroblastoma in Second or Later Remission

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Abstract

**Purpose:** To report on a phase I trial designed to find the maximally tolerated dose in children of the immunologic adjuvant OPT-821 in a vaccine containing neuroblastoma-associated antigens (GD2 and GD3; Clinicaltrials.gov NCT00911560). Secondary objectives were to obtain preliminary data on immune response and activity against minimal residual disease (MRD). Treatment also included the immunostimulant β-glucan.

**Experimental Design:** Patients with neuroblastoma in ≥2nd complete/very good partial remission received vaccine subcutaneously (weeks 1–2–3–8–20–32–52). Vaccine contained 30 μg each of GD2 and GD3 stabilized as lactones and conjugated to the immunologic carrier protein keyhole limpet hemocyanin; and OPT-821, which was dose escalated as 50, 75, 100, and 150 mg/m² per injection. Oral β-glucan (40 mg/kg/day, 14 days on/14 days off) started week 6.

**Results:** The study was completed with 15 patients because there was no dose-limiting toxicity at 150 mg/m² of OPT-821 (the dosing used in adults). Thirteen of fifteen patients received the entire protocol treatment, including 12 who remain relapse-free at 24+ to 39+ (median 32+) months and 1 who relapsed (single node) at 21 months. Relapse-free survival was 80% ± 10% at 24 months. Vaccine and β-glucan were well tolerated. Twelve of fifteen patients had antibody responses against GD2 and/or GD3. Disappearance of MRD was documented in 6 of 10 patients assessable for response.

**Conclusions:** This immunotherapy program lacks major toxicity and is transportable to any outpatient clinic. Patient outcome is encouraging but the efficacy is uncertain because of the complexity and heterogeneity of prior therapies. A larger phase II trial is underway. Clin Cancer Res; 20(5); 1375–82.
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Introduction

Relapse of high-risk neuroblastoma (HR-NB) has long been viewed as tantamount to eventual death from progressive disease or toxicity (1). We hypothesize that active humoral immunity could maintain second or subsequent remission even among these ultra-high-risk patients. This curative approach starts from diagnosis and has several components. First, patients in initial remission undergo close monitoring, with extent-of-disease evaluations at least every 3 months through ≥3 years from diagnosis; the aim is to detect focal relapse early, which may be more amenable to successful salvage than a widespread relapse with a large tumor burden (2). Second, relapse is treated with a multimodality program that comprises surgery and/or radiotherapy for local control, plus salvage chemotherapy combining agents with well-established anti-neuroblastoma activity and tailored to the patient’s particular clinical context or past treatment history (3–8). Third, after achieving a maximal response with the standard modalities (chemotherapy, radiotherapy, surgery), remission is consolidated via immunotherapy using monoclonal antibody (MoAb) or vaccine, the latter being the subject of this report.

The vaccine is designed to induce antibodies against gangliosides GD2 and GD3, which are highly expressed on neuroblastoma. Indeed, the intensity of ganglioside expression on neuroblastoma is unique. Although melanoma (considered a ganglioside-rich tumor) yields a median of 43 mcg of GD3 per gram of tissue biopsied and 3.1 mcg of GD2, neuroblastoma yields a similar level of GD3 but 10× higher levels of GD2 (44 and 31 mcg, respectively; refs. 9 and 10). GD2 and GD3 are also expressed on peripheral nerves and brain, and treatment with anti-GD2/GD3 MoAbs causes pain (11–13), but spares the central nervous system, presumably because of the blood–brain barrier.

Preclinical studies and clinical trials of vaccines in adults showed immunogenicity significantly augmented by (1) stabilizing GD2 and GD3 through lactone formation; (2)
Translational Relevance

In preclinical models and clinical trials, immunotherapy using anti-GD2 or anti-GD3 monoclonal antibodies (MoAbs) has proven beneficial against high-risk neuroblastoma (HR-NB) and melanoma. This article presents results of a phase I study of a vaccine designed to induce host anti-ganglioside antibodies that can replicate the antineoplastic activities of intravenously administered MoAbs. Patients also received β-glucan, which synergizes with anti-GD2/GD3 MoAbs. The vaccine/β-glucan treatment was well tolerated, and serological and minimal residual disease (MRD) responses were encouraging. Multiple factors may account for the excellent relapse-free survival despite prior relapse, which historically conferred a dismal prognosis. This phase I experience could have a major impact on treatment of HR-NB. Thus, a phase II trial is already in progress; the results might support vaccine/β-glucan as adjuvant after completion of the standard upfront HR-NB multimodality program. The latter now includes anti-GD2 MoAb but additional nontoxic therapy for MRD is eagerly sought, to help further improve outcome.

conjugating the resulting GD2L (L for lactone) and GD3L structures to the immunologic carrier protein keyhole limpet hemocyanin (KLH); and (3) combining GD2L-KLH and GD3L-KLH with the immunologic saponin adjuvant QS-21 (14–19). For both GD2L and GD3L, the lowest optimal dose was 30 mcg per vaccine (18,19). For Q5-21, doses of 50 to 200 mcg were tested and found safe, with side effects of grade 3 erythema and flu-like symptoms lasting 2 to 4 days (20). When QS-21 became unavailable, we turned to OPT-821, which is a QS-21 equivalent based on 1H and 13C nuclear magnetic resonance, mass spectrometry, high-performance liquid chromatography studies, and overlapping immunogenicity and toxicity profiles in mice.

We now report a phase I trial involving children with HR-NB in second or later remission, treated with a GD2/GD3 vaccine containing OPT-821. Patients also received β-glucan, which has a broad range of immunostimulant effects (21–23) and synergizes with anti-GD2/GD3 MoAbs in preclinical studies (24,25). β-Glucans bind to CR3 (Mac-1), a complement receptor for iC3b, widely expressed on leukocytes (21). This receptor mediates the diapedesis of leukocytes through the endothelium and stimulates phagocytosis, degranulation, and tumor cytotoxicity. Many bacteria and fungi carry activating ligands for CR3, which renders them vulnerable to phagocytosis by neutrophils. Human tumors lack such molecules and CR3-mediated tumor lysis is suboptimal until it is activated by GM-CSF (26) or by ligands such as β-glucan (27).

Materials and Methods

The primary objective of the Memorial Sloan-Kettering Cancer Center (MSKCC) protocol 05-075 phase I trial (Clinicaltrials.gov NCT00911560) was to find the maximally tolerated dose (MTD) in children of the immunologic adjuvant OPT-821 in a vaccine containing 2 neuroblastoma-associated antigens (GD2 and GD3). Secondary objectives were to obtain preliminary data on anti-GD2 and anti-GD3 immune response and anti-neuroblastoma activity against minimal residual disease (MRD).

Patient selection

This study enrolled patients ≤21 years old with HR-NB who had previously relapsed but had achieved a ≥2nd complete/very good partial remission (CR/VGPR). Disease status was confirmed ≥3 weeks after other therapy by computed tomography, magnetic resonance imaging (MRI), 123I-metaiodobenzylguanidine (MIBG) scan, bone marrow histology (aspirates and biopsies from bilateral posterior and anterior iliac crests), and urine catecholamines. Using international criteria (28) expanded to include 123I-MIBG findings, complete remission was defined as absence of neuroblastoma by all studies, and VGPR was defined as the primary mass reduced ≥90%, no evidence of active distant disease, and urine catecholamines normal. There were no eligibility criteria/limits about prior therapy. Major organ toxicity was required to be ≤grade 3, except neurologic status had to be ≤grade 1, by the Common Terminology Criteria for Adverse Events, Version 3.0 (CTCAEv3.0), and the absolute lymphocyte and neutrophil counts each had to be ≥500/μL. Informed written consents for treatment and tests were obtained according to MSKCC Institutional Review Board rules.

Study design

Treatment included 7 subcutaneous injections of the vaccine administered weeks 1–2–3–4–8–20–32–52 (Fig. 1). The vaccine contained 30 μg of GD2L and 30 μg of GD3L, each conjugated to KLH, for all patients, but OPT-821 dosing depended on dosage level and body-surface area. Four OPT-821 dosage levels were planned: 50, 75, 100, and 150 μg/m2; the dose was “capped” with the maximal dose being 75, 110, 150, or 200 μg at the respective dosage levels. Oral β-glucan was started week 6 (Fig. 1), to allow time for generation of antibodies. Dosage (40 mg/kg/day, 14 days on/14 days off) was identical for all patients and derived from the phase I MSKCC protocol 05-073 (Clinicaltrials.gov NCT00492167). β-Glucan [a botanical extract of sugar polymer consisting of (1 → 3),(1 → 6)-β-glycosidic linkage from baker’s yeast, manufactured by Biotec Pharmacon ASA

Figure 1. Protocol treatment schema. Glucan, β-glucan from yeast; Vac, GD2–GD3 vaccine containing OPT-821.
Vaccine and Glucan for Neuroblastoma

Materials

GD2 and GD3 were obtained from Matreya Inc., with GD2 prepared via extraction of GD1b from rabbit brain followed by treatment with bull testis β-galactosidase (19), and GD3 extracted from bovine buttermilk (18). GD2 and GD3 were >95% pure as determined by thin-layer chromatography and instant thin-layer chromatography (ITLC). Clinical grade KLH was acquired from Sigma Chemicals Inc., and clinical grade OPT-821 from Optimer Pharmaceuticals Inc.

For conjugation of gangliosides to KLH, ozone was used to cleave the ceramide double bond, an aldehyde group was introduced, and this product was coupled to amino groups based on KLH by reductive amination as described (18). The ratio of antigen to KLH in the final conjugates was determined using the resorcinol method for measuring sialic acid and the Bio-Rad dye-binding method. The percent unconjugated antigen in the final vialed conjugates was determined by the Bio-Rad dye-binding method. The percent unconjugated antigen in the final vialed conjugates was determined by the Bio-Rad dye-binding method. The percent unconjugated antigen in the final vialed conjugates was determined by the Bio-Rad dye-binding method. The percent unconjugated antigen in the final vialed conjugates was determined by the Bio-Rad dye-binding method.

Vaccine preparation. On the day of vaccination, the prescribed dose +10% of OPT-821 was diluted with normal saline to a final volume of 1.1 or 1.2 mL for the 33 or 36 mg/kg dose of OPT-821. Antigen was permitted. This vaccine was used under an IND held by MSKCC, BBIND 4976. GD2 and GD3 were obtained from Matreya Inc., with GD2 prepared via extraction of GD1b from rabbit brain followed by treatment with bull testis β-galactosidase (19), and GD3 extracted from bovine buttermilk (18). GD2 and GD3 were >95% pure as determined by thin-layer chromatography and instant thin-layer chromatography (ITLC). Clinical grade KLH was acquired from Sigma Chemicals Inc., and clinical grade OPT-821 from Optimer Pharmaceuticals Inc.

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Vaccine injections were performed if liver function tests were ≤ grade 1 toxicity. For grade 2 or 3 toxicity, injections were deferred until a return to ≤ grade 1.

A standard dose-escalation schema was used: escalations were implemented if 0 of 3 or ≤1 of 6 patients at a given dosage level had DLT. At least 6 patients were studied at the MTD, or at the highest planned level (i.e., 150 mcg/m²) if there were no DLTs.

Results

Patient characteristics

The study was completed with 15 patients (enrolled July 2009–November 2010) because there was no DLT and OPT-821 dosing reached that used in adults (Table 1). At enrollment, patients were 3.3 to 16.7 (median 8.1) years old. All patients had stage 4 neuroblastoma. 11 were in 2nd and 4 in ≥3rd CR/VGPR. Five (33%) patients had MYCN-amplified neuroblastoma. The time from diagnosis to first
<table>
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<tr>
<th>Patient No.</th>
<th>Dose level*</th>
<th>MYCN amplified?</th>
<th>Time to first relapse</th>
<th>Sites of relapse</th>
<th>Treatment of relapse</th>
<th>Time from relapse to vaccine</th>
<th>MRD in BM pre/post</th>
<th>Serologic responseb to:</th>
<th>Outcome (time from first vaccine)</th>
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<td></td>
<td></td>
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<td>1</td>
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<td>Brain, abdomen</td>
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<td>Abdomen</td>
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<td>+/−</td>
<td>+ (4)</td>
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<td>2</td>
<td>No</td>
<td>23 mo</td>
<td>Abdomen</td>
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<td>−/−</td>
<td>−</td>
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<td>−</td>
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<td>8 mo</td>
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<td>−</td>
<td>(7) RFS (33 mo)</td>
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<td>26 mo</td>
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<td>9 mo</td>
<td>+/−</td>
<td>+ (4)</td>
<td>(3)PT-821</td>
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<td></td>
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<tr>
<td>12</td>
<td>1</td>
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<td>15 mo</td>
<td>Thorax</td>
<td>GTR, RT, CPT/TMZ(×9), 3F8, CRA, Thalidomide-Celecoxib</td>
<td>15 mo</td>
<td>−/−</td>
<td>+ (3)</td>
<td>(7) RFS (48 mo)</td>
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<td>47 mo</td>
<td>Thorax</td>
<td>Cyclo-Topo(×8), Lenalidomide-Celecoxib</td>
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<td>+/−</td>
<td>−</td>
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<td>21 mo</td>
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<td>+ (4)</td>
<td>(4) PT-821</td>
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<tr>
<td>15</td>
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<td>17 mo</td>
<td>R supra-clavicular</td>
<td>131I-MIBG, RT</td>
<td>3 mo</td>
<td>+/−</td>
<td>(7)</td>
<td>(42 mo)</td>
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Abbreviations: BM, bone marrow; CAV, high-dose Cyclo-doxorubicin-vincristine; CCV, high-dose Cyclo-PCT-vincristine (7); CIT, high-dose carboplatin-CPT-TMZ (6); CPT, irinotecan; CRA, 13-cis-retinoic acid; CTV, high-dose Cyclo-Topo-vincristine (5); Cyclo, cyclophosphamide; DoD, died of disease; GTR, gross total resection; ICE, high-dose ifosfamide-carboplatin-etoposide (8); IT-3F8, intrathecal 131I-3F8 (4); MRD, minimal residual disease; n.d., not done; P/E, cisplatin-etoposide; RFS, relapse-free survival; Ritux, rituximab; RT, local radiotherapy; TMZ, temozolomide; Topo, topotecan.

*aOPT-821 dose level 1 = 50 μg/m², dose level 2 = 75 μg/m², dose level 3 = 100 μg/m², and dose level 4 = 150 μg/m².

bNumber of prior vaccine injections on this protocol is in parentheses.
relapse was <12 months (n = 2), 12 to 18 months (n = 5), 19 to 24 months (n = 4), and >24 months (n = 4). Retrieval therapy before study enrollment included local control with radiotherapy alone (n = 7), radiotherapy plus surgery (n = 7), strongly myelosuppressive chemotherapy (n = 8),5–8 and nonimmunosuppressive treatments such as irinotecan-temozolomide (n = 9; refs. 3 and 4)3,4 and anti-GD2 MoAb (13; n = 5); no patient underwent stem-cell transplantation as part of retrieval. These retrieval treatments were for relapses that were localized in 10 patients, including soft tissue (n = 7) or osteomediulary by MIBG and MRI (n = 3), and widespread in 5 patients, including bone marrow by histology and MIBG scan (n = 2), multifocal MIBG osteomediulary with (n = 1) or without (n = 1) soft tissue, and retroperitoneal nodes plus brain (n = 1).

Survival and toxicity
Thirteen of fifteen patients received all 7 protocol-prescribed injections of vaccine, including 12 patients who remain relapse-free at 21+ to 36+ (median 29+) months and one who had a focal relapse (supracavicular node) at 21 months. Two patients had early focal relapses (2.3 and 4.6 months). All 3 relapses were among patients receiving 100 to 150 μg/m² of OPT-821, including 1 with MYCN-amplified and 2 with MYCN-nonamplified neuroblastoma. RFS was 87% ± 9% at 12 and 80% ± 10% at 24 months (Fig. 2). Overall survival was 93% ± 6% (follow-up >25 months). One relapse-free survivor received a cycle of chemotherapy because of radiologic findings deemed suspicious for soft tissue relapse of his MYCN-amplified neuroblastoma; laparotomy revealed no evidence of neuroblastoma, so patient was allowed to resume the vaccine/β-glucan immunotherapy.

Treatment was well tolerated, with no DLT, delays in administration of vaccine, co-interventions, or neurologic sequelae. There was no relation between OPT-821 dosage and side effects. Injections were painful and elicited grade 1 local reactions in 12 patients (varying from 1 to 4 injections per patient) and a grade 2 local reaction in one patient (dose level 1, with injection #7); these side effects subsided within 24 hours. No other grade 2 toxicities of protocol treatment were noted. One patient had a grade 3 elevation in liver function enzymes on day 5 after injection #2 (dose level 1). The only other grade 3 to 4 toxicities—all self-limited and attributable to prior therapy or intercurrent events—were concurrent hypocalcemia and hypokalemia (1 patient), lymphopenia (1 patient), and neutropenia (1 patient).

Serological response
Patient serum for assessment of anti-GD2 and anti-GD3 titers was collected before vaccine injection #1 and after the subsequent injections. Seropositive response was defined as negative titer at baseline, and positive titer anytime, whereas seronegative response was defined as negative titer at baseline and all subsequent time points tested. Three of fifteen patients had seronegative response. The rest of them became seropositive against GD2 alone (n = 2), GD3 alone (n = 3), or both (n = 7; Table 1). Anti-GD2 seropositivity was documented after cycles #2 (n = 1), #3 (n = 2), #4 (n = 5), and #7 (n = 1). Anti-GD3 seropositivity was documented after cycles #3 (n = 2), #4 (n = 2), #6 (n = 2), and #7 (n = 4) (Fig. 3). Seronegativity did not correlate with relapse.

MRD response
At study enrollment, all 15 patients had complete remission in bone marrow by histology. Response with respect to MRD was assessed by comparing bone marrow collected at baseline before vaccine injection #1 versus after the last injection. Of 11 patients who were MRD positive at baseline, 6 became MRD negative, 4 remained MRD positive, and 1 patient in continual remission was not retested for MRD because there was no available sample (Table 1). Four patients were MRD negative at baseline and remained MRD negative. MRD nonresponse did not correlate with relapse.

Discussion
This phase I trial in patients with HR-NB in ≥2nd CR/VGPR assessed the toxicity of escalating dosages of the immunologic adjuvant OPT-821 in a bivalent vaccine containing GD2L and GD3L, each covalently attached to the immunologic carrier protein KLH. The patients also took the oral immunostimulant β-glucan. Because thirteen of fifteen study patients received all 7 injections of the protocol, the study’s primary objective about safe dosing of OPT-821 was achieved. Treatments were devoid of acute toxicities aside from local reactions immediately postinjection. No delayed toxicities developed, alleviating concerns about neurotoxicity given GD2/GD3 expression on peripheral nerves and in brain. Prolonged oral intake of β-glucan by the children in this study was uncomplicated, consistent with the absence of major side effects in preclinical animal models and in human adults.

Although the absence of toxicity with the bivalent vaccine and β-glucan seems well established, anti-neuroblastoma efficacy should be interpreted with great caution. The study patients achieved excellent RFS (Fig. 2), in contrast to dismal predictions based on the international experience with relapsed HR-NB (31–33), and without myeloablative therapy with autologous stem-cell transplantation, which is widely used as consolidation of first or subsequent remissions. Yet the contributory role of, for example, β-glucan to
RFS is uncertain, and major confounding factors included patient selection and the proximity and complexity of therapy before vaccine treatment. Nevertheless, twelve of fifteen patients mounted an anti-disialoganglioside immune response, and bone marrow MRD response (from positive to negative) occurred in 60% of evaluable patients. All 3 patients who progressed had complete remission of bone marrow MRD. It is of note that 2 patients had isolated progression (1 in a neck node and 1 in left ichium) with continual marrow remission for 17 months and 24 months. The third patient progressed with a new paraspinal soft tissue mass with only 1 of 6 marrow samples turning positive, and had negative marrow aspirates and biopsies (total of 60 samples) alive and well at 41 months since.

Vaccine therapy in neuroblastoma has previously focused on T-cell immunity. Syngeneic or allogeneic neuroblastoma transfected with interleukin-2 can stimulate anti-neuroblastoma IgG (35). Allogeneic neuroblastoma transfected with both interleukin-2 and lymphotactin has induced major responses in patients (36,37). Dendritic cells expanded in vitro and "pulsed" with crude cell lysates or tumor RNA have also been tested in children (38,39). Unfortunately, to date, specific CTLs (e.g., against survivin) have not been shown to home to neuroblastoma in patients (40). Furthermore, T-cell immunity has been limited by the state of immunosuppression following dose-intensive chemotherapy. In this study, the successful induction of anti-GD2 and anti-GD3 antibodies using lactone-KLH in the presence of OPT-821 in patients despite prior history of immunosuppressive chemotherapy is encouraging.

In preclinical models (41,42) and clinical trials (11–13), immunotherapy using anti-GD2 or anti-GD3 MoAbs has shown activity against neuroblastoma and melanoma. Benefit is optimal in the adjuvant setting, with ablation of micrometastasis (43). The antineoplastic mechanisms include opsonization, antibody-dependent cellular cytotoxicity (ADCC), and complement-mediated cytotoxicity (CMC). The ADCC involves leukocytes of all types, including neutrophils, macrophages, and natural killer cells. Our

![Figure 3. Antibody response against GD2 in ng/mL (y-axis) after each vaccine cycle number (x-axis) for individual patients who turned seropositive, grouped according to the dose level of OPT-821 (50, 75, 100, and 150 µg/m²). At 100 µg/m² dose level, one patient (dash) had unusually high antibody titer plotted on the secondary y-axis. Patients who did not mount any anti-GD2 antibody response were not included in these plots (1/3 patients at 75 µg/m² dose level and 4/6 patients at 150 µg/m² dose level). The 3 patients who progressed were marked with black arrows.](image-url)
bivalent vaccine is constructed with the purpose of inducing host anti-ganglioside antibodies that can replicate the anti-neoplastic effects of exogenously administered MoAbs.

Various strategies have undergone clinical testing to induce or augment immune-mediated attack against cancer (44). Few clinical trials, however, have used humoral vaccines against solid tumors in children, especially with ADCC and CMC as the principal underlying immunocytotoxic mechanisms (45). Also, an anti-neoplastic role for myeloid cells (e.g., neutrophils) has received scant attention (27). A treatment program combining an anti-neuroblastoma vaccine with an agent that enhances granulocyte cytotoxicity (i.e., β-glucan) represents a novel strategy to maximize this myeloid effect in the emerging field of immunotherapy.

The setting of this protocol is an ideal model system to assess anticancer vaccine therapy: patients with a low disease burden, but at great risk for relapse; target antigens highly expressed on the cancerous but not normal tissues; immunological adjuvants (KLH and OPT-821) to optimize induction of antibodies against the target antigens (GD2 and GD3); and a nontoxic oral agent (β-glucan) to enhance effector cytotoxicity. The basis for emphasis on multivalent vaccines is tumor-cell heterogeneity, variability of the immune response among patients, and the correlation between the concentration of tumor-selective antibody and effector mechanisms (45). In the adjuvant setting, prolonged presence of anti-neoplastic antibodies should be more efficient against MRD. Maintenance of antibodies over months or years is more readily achieved with vaccines than MoAbs where repeated administrations are necessary and human anti-mouse or anti-human blocking antibodies can develop. We speculate that the fluctuations of anti-GD2 titer in vaccinated patients could represent multiple idiotypes within an idiotypic network whereas unimodal anti-GD2 response could represent ineffective idiotypes associated with an unfavorable outcome (46–48). Alternatively, these fluctuations could represent random oscillations of unknown significance.

In conclusion, the bivalent-vaccine/β-glucan immunotherapy program engenders no major toxicity, even in heavily prior-treated children, and is readily transportable to multiple institutions. Patients have no neuropathic pain, unlike patients receiving intravenous anti-GD2 or anti-GD3 MoAbs. Serological and MRD responses were encouraging. Multiple factors may account for the excellent RFS. This limited experience provides support for pursuing an aggressive curative strategy against relapsed HR-NB. In addition, unlike most pediatric phase I studies, this one could have a major impact on treatment of HR-NB. Thus, a phase II trial is already in progress, using the phase I study’s maximum OPT-821 dosage (150 μg/m²); the results might support vaccine-glucan as adjuvant after completion of the standard upfront HR-NB multimodality program. The latter now includes anti-GD2 MoAb (12) but additional nontoxic therapy for MRD is eagerly sought, to help further improve outcome.

Disclosure of Potential Conflicts of Interest
G. Ragupathi has ownership interest (including patents) in MabVax Therapeutics. G. Ragupathi is a consultant/advisory board member of MabVax Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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