Anti-GD2 antibody 3F8 and barley-derived (1 → 3),(1 → 4)-β-D-glucan: A Phase I study in patients with chemoresistant neuroblastoma

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β-glucans are complex, naturally-occurring polysaccharides that prime leukocyte dectin and complement receptor 3. Based on our preclinical findings, indicating that oral barley-derived (1 → 3),(1 → 4)-β-D-glucan (BG) synergizes with the murine anti-GD2 antibody 3F8 against neuroblastoma, we conducted a Phase I clinical study to evaluate the safety of this combinatorial regimen in patients affected by chemoresistant neuroblastoma. In this setting, four cohorts of six heavily pre-treated patients bearing recurrent or refractory advanced-stage neuroblastoma were treated with 3F8 plus BG. Each cycle consisted of intravenous 3F8 at a fixed dose of 10 mg/m²/day plus concurrent oral BG, dose-escalated from 10 to 80 mg/Kg/day, for 10 d. Patients who did not develop human anti-mouse antibodies could be treated for up to 4 cycles. Twenty-four patients completed 50 cycles of therapy. All patients completed at least one cycle and were evaluable for the assessment of toxicity and responses. The maximum tolerated dose of BG was not reached, but two patients developed dose-limiting toxicities. These individuals developed grade 4 thrombocytopenia after one cycle of BG at doses of 20 mg/Kg/day and 40 mg/Kg/day, respectively. Platelet counts recovered following the administration of idiopathic thrombocytopenic purpura therapy. There were no other toxicities of grade > 2. Thirteen out of 22 patients with pre-treatment positive 123I-MIBG scans demonstrated clinical improvement on semiquantitative scoring. Responses did not correlate with BG dose or with in vitro cytotoxicity. In summary, 3F8 plus BG is well tolerated and shows antineoplastic activity in recurrent or refractory advanced-stage neuroblastoma patients. Further clinical investigation of this novel combinatorial immunotherapeutic regimen is warranted.

Introduction

A majority of patients with neuroblastoma (NB), the most common extracranial solid tumor of children, relapse and die despite aggressive multimodality therapy.1–4 Antibody-mediated anti-GD2 immunotherapy has emerged as an exciting addition to the standard management of NB, as demonstrated in a Phase III study.5 However, for efficacy, this approach requires the concomitant administration of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-2, the latter being associated with significant toxicity.6 Furthermore, anti-GD2 monoclonal antibodies (mAbs) exert modest antineoplastic activity in patients bearing chemoresistant NB exceeding the minimal residual disease.7 The anti-GD2 murine mAb 3F8 activates the complement system4 and mediates NB cytotoxicity by human lymphocytes,5 cultured monocytes10 and granulocytes.11 3F8 binds the FcyRII and FcyRIII Fc receptors and exploits the GM-CSF-mediated activation of CD11b for eliciting antibody-dependent cellular cytotoxicity (ADCC) by myeloid cells.12,13 3F8 has been studied in Phase I and II trials, with demonstrated safety, and exerts antineoplastic activity in NB patients.14–17

β-glucans are complex glucose polymers containing a backbone of β-1,3-linked and β-1,4-D-glucose molecules.18 β-glucans are found in food including cereals, mushrooms and yeast, as well as in some bacteria. The first clinical studies of β-glucans as antitumor agents were reported by Japanese investigators in the 1980s, although preclinical investigations have been ongoing for nearly 40 years.19 The mushroom-derived (1 → 3),(1 → 6)-β-D-glucans, protein-bound polysaccharide and schizophyllan modestly improved the survival rate of patients with gastrointestinal cancer.20,21 Conversely, barley-derived (1 → 3),(1 → 4)-β-D-glucans have undergone clinical testing primarily for their cholesterol-reducing activity.22
Glucan-binding receptors on human leucocytes include lactosylceramide,23 lectin-124 and the complement receptor 3 (CR3).25 Most recent studies indicate that CR3 is the major receptor mediating the immunological effects of β-glucans.26,27 mAbs allow for the fixation of the complement factor C3b on tumor cells, and the proteolytic fragment iC3b binds CR3 (also known as CD11b/CD18, Mac 1 or α5β2 integrin), which is expressed on the surface of neutrophils, monocytes, macrophages and natural killer (NK) cells. The activation of CR3, which facilitates diapedesis, phagocytosis and degranulation,28,29 requires the engagement of two sites on its α subunit (CD11b): an iC3b-binding site within the I domain at the N-terminus and a lectin site at the C-terminus, which is bound by β-glucans.30 Of note, iC3b-opsonized tumor cells cannot activate leukocytes unless the C-terminus of CR3 is bound by β-glucans.31,32 Along similar lines, in pre-clinical models, tumors fail to respond to β-glucans in the absence of complement-fixing anti-tumor mAbs (as demonstrated in SCID mice), C3 or leukocyte CR3 (as demonstrated in knockout mouse models), highlighting the essential components of this iC3b-based cytotoxic strategy.32 Although barley-derived (1 → 3),(1 → 4)-β-D-glucan (BG) binds to CR333 and activates ADCC in vitro,25,34,35 most previous studies of the immunomodulatory activity of β-glucans in cancer have focused on mushroom or yeast-derived (1 → 3),(1 → 6)-β-D-glucan.36 In xenograft models, the antitumor effect of complement-activating mAbs was enhanced by oral BG,37,38 as well as by (1 → 3),(1 → 6)-β-D-glucan from yeast or mushrooms.39 Here, we report the results of a Phase I clinical study involving BG in combination with the anti-GD2 mAb 3F8 in patients with chemoresistant metastatic NB.

Results

Patients. Twenty-four heavily-pretreated patients (13 males, 11 females), with a median age of 8 y (range: 35 mo to 19 y), entered the study, 8 with progressive disease (PD), and 16 with stable disease (SD) (Table 1). Previous therapeutic regimens included stem-cell transplantation, in 16 patients. A total of 50 cycles of 3F8 plus BG were administered, with patients

Table 1. Patient characteristics and toxicities

| Pt. no. | Age at Rx (yrs) | Dose level (mg/kg/day) | No. of prior regimens | Pre-Rx status | Pre-therapy extent of disease | No. of cycles | Reason for withdrawal | Unexpected therapy-related toxicities |
|---------|-----------------|-----------------------|-----------------------|---------------|-------------------------------|---------------|-----------------------|----------------------------------------|
| 1       | 14              | 10                    | 3                     | SD            | MIBG, BM                      | 2             | HAMA                  | None                                   |
| 2       | 17              | 10                    | 5                     | SD            | MIBG, CT                      | 3             | PD                    | None                                   |
| 3       | 4               | 10                    | 3                     | SD            | MIBG, BM, CT                  | 2             | HAMA                  | None                                   |
| 4       | 5               | 10                    | 2                     | SD            | MIBG                          | 4             | Completed             | None                                   |
| 5       | 8               | 10                    | 3                     | SD            | MIBG, BM                      | 3             | PD                    | None                                   |
| 6       | 7               | 10                    | 3                     | PD            | MIBG, CT                      | 1             | PD                    | None                                   |
| 7       | 10              | 20                    | 3                     | SD            | MIBG, BM                      | 2             | HAMA                  | None                                   |
| 8       | 15              | 20                    | 3                     | SD            | MIBG, BM                      | 4             | Completed             | None                                   |
| 9       | 9               | 20                    | 6                     | PD            | MIBG, CT, BM                  | 1             | HAMA                  | None                                   |
| 10      | 4               | 20                    | 3                     | PD            | CT, PET                       | 1             | PD                    | None                                   |
| 11      | 4               | 20                    | 2                     | PD            | MIBG, CT, BM                  | 1             | PD                    | None                                   |
| 12      | 6               | 20                    | 3                     | SD            | MIBG, BM                      | 1             | DLT                   | Grade 4 immune thrombocytopenia         |
| 13      | 2               | 40                    | 2                     | PD            | MIBG, CT, BM                  | 4             | DLT                   | Grade 4 immune thrombocytopenia         |
| 14      | 4               | 40                    | 2                     | SD            | MIBG, BM, CT                  | 1             | DLT                   | None                                   |
| 15      | 6               | 40                    | 6                     | SD            | MIBG, CT                      | 1             | DLT                   | None                                   |
| 16      | 18              | 40                    | 3                     | SD            | MIBG, CT                      | 1             | HAMA                  | None                                   |
| 17      | 17              | 40                    | 5                     | SD            | MIBG, BM                      | 1             | HAMA                  | None                                   |
| 18      | 9               | 40                    | 5                     | PD            | MIBG, BM, CT                  | 2             | PD                    | None                                   |
| 19      | 12              | 80                    | 3                     | SD            | MIBG, CT                      | 4             | Completed             | None                                   |
| 20      | 5               | 80                    | 2                     | SD            | MIBG, BM                      | 1             | PD                    | None                                   |
| 21      | 3               | 80                    | 4                     | PD            | MIBG, CT, BM, PET             | 1             | PD                    | None                                   |
| 22      | 14              | 80                    | 2                     | SD            | MIBG, BM                      | 4             | Completed             | None                                   |
| 23      | 3               | 80                    | 2                     | PD            | MIBG, CT                      | 1             | PD                    | None                                   |
| 24      | 9               | 80                    | 2                     | SD            | MIBG, BM                      | 4             | Completed             | None                                   |

Abbreviations: BM, evidence of disease on bone marrow aspirate and/or biopsy; CT, measurable soft tissue disease on CT; DLT, dose limiting toxicity; HAMA, human anti-mouse antibody response; MIBG, evaluable disease on 123I-MIBG scan; PD, progressive disease; PET, evaluable disease on FDG PET scan; Pt, patient; Rx, 3F8/BG therapy; SD, stable disease; yrs, years.
receiving 1 (n = 12), 2 (n = 4), 3 (n = 2) or 4 (n = 6) cycles. Patients were taken off study due to the development of human anti-mouse antibody (HAMA) responses after cycle 1 (n = 3) or cycle 2 (n = 3); owing to PD after cycle 1 (n = 7), cycle 2 (n = 1) or cycle 3 (n = 2); or upon dose-limiting toxicities (DLTs) (n = 2).

**Toxicities.** Most patients tolerated oral BG well. Patients experienced 3F8-related pain, fever and urticaria. In addition, 2 patients manifested a DLT previously unseen with 3F8-based therapy. Both these patients developed grade 4 acute thrombocytopenia featuring increased bone marrow (BM) megakaryocytes after developing HAMA responses, immediately after cycle one. Neither of these patients was retreated. Thrombocytopenia responded to therapy for idiopathic thrombocytopenic purpura (ITP), consisting of a single administration of dexamethasone, vincristine, anti-D and/or intravenous immunoglobulin (IVIG). One of these 2 patients had a complete resolution of the thrombocytopenia without recurrence (#15), while the other (#12) developed chronic thrombocytopenia despite initially responding to ITP therapy. To maintain platelet counts, the latter required intermittent ITP therapy until death (owing to progressive NB). There were no other toxicities > grade 2. The maximum tolerated dose (MTD) of BG was not reached: the 2 patients developed thrombocytopenia at BG doses of 20 mg/Kg/day and 40 mg/Kg/day, respectively.

**Disease responses.** All patients could be assessed for clinical response, which was a secondary objective of this study. Overall, 13/24 (54%) patients demonstrated PD while 11/24 (46%) had stable disease. Although no patient achieved partial response (PR) or better, objective responses (ORs) were observed in several patients. Improvement in 123I-meta-iodobenzylguanidine (MIBG) scans occurred in 13/22 (59%) patients, with mean reductions in Curie extension scores for the entire group and responders being 2.6 and 3.8, respectively (reduction range 1 to 12) (**Table 2**). There was no dose-response benefit for BG (p = 0.39 for MIBG responses, comparing dose level 1 and 4 by Fisher’s exact test). Two patients had a near complete remission of skeletal MIBG uptake (**Fig. 1**). Five out of 15 (33%) evaluable patients with BM disease manifested CRs in the BM. These responses were transient, except in 1 patient (#7). Soft tissue disease reduced slightly in 1 (7%) out of 14 patients, but otherwise remained unchanged (n = 7) or increased (n = 6). Of 11 patients exhibiting elevated urinary catecholamines at baseline, 6 had this parameter reduced,

| Pt. No. | Dose level mg/kg/day | Best response (after cycle no.) | Change in semiquantitative MIBG extension score | Overall response (after cycle no.) |
|---------|----------------------|---------------------------------|-----------------------------------------------|----------------------------------|
| 1   | 10                    | MIBG improved (1)               | -1                                            | PD (2)                           |
| 2   | 10                    | MIBG improved* (1)              | -1                                            | PD (3)                           |
| 3   | 10                    | MIBG improved                  | -12                                           | SD (4)                           |
| 4   | 10                    | MIBG improved (4)               | -7                                            | SD (4)                           |
| 5   | 10                    | MIBG improved, BM not evaluable (1) | -10                                         | PD (3)                           |
| 6   | 10                    | PD (1)                          | 0                                             | PD (1)                           |
| 7   | 20                    | MIBG unchanged, BM CR (1)      | -3                                            | SD; BM CCR (2)                   |
| 8   | 20                    | MIBG and BM unchanged          | 0                                             | PD (4)                           |
| 9   | 20                    | MIBG improved (1)               | -3                                            | SD (1)                           |
| 10  | 20                    | PD (1)                          | +4                                            | PD after 1                       |
| 11  | 20                    | PD (1)                          | NE                                            | PD after 1                       |
| 12  | 20                    | MIBG improved, BM CR (1)       | -4                                            | SD (1)                           |
| 13  | 40                    | PD (1)                          | +1                                            | PD (1)                           |
| 14  | 40                    | MIBG, CT improved, BM CR (1)   | -8                                            | SD (4)                           |
| 15  | 40                    | MIBG improved (1)               | -1                                            | SD (1)                           |
| 16  | 40                    | MIBG improved (1)               | -1                                            | SD (1)                           |
| 17  | 40                    | MIBG improved (1)               | -1                                            | SD (1)                           |
| 18  | 40                    | MIBG unchanged; BM CR (1)      | 0                                             | PD (2)                           |
| 19  | 80                    | MIBG unchanged; SD (1)          | 0                                             | PD (4)                           |
| 20  | 80                    | MIBG unchanged; SD (1)          | 0                                             | PD (1)                           |
| 21  | 80                    | PD (1)                          | +1                                            | PD (1)                           |
| 22  | 80                    | MIBG improved, BM CR (4)        | -11                                           | SD (4)                           |
| 23  | 80                    | PD (1)                          | NE                                            | PD (1)                           |
| 24  | 80                    | MIBG improved (2)               | -4                                            | SD (4)                           |

*Abbreviations: BM, bone marrow; CCR, continued complete remission; CR, complete remission; MIBG, 123I-MIBG scan; mo, months; NE, not evaluated; PD, progressive disease; SD, stable disease. Durations calculated from start of therapy with 3F8/BG. *Pt No. 2 had received radiation therapy to site of skeletal MIBG uptake about 4 weeks prior to 3F8/BG.*
increase in dose was 1.12 (95% CI 0.73, 1.73; p = 0.6). The development of HAMAs was associated with improved OS (HR = 0.32; 95% CI 0, 9.47; p < 0.001), the median survival of patients who did not develop HAMAs (n = 12) being 25 mo and that of patients who did develop HAMAs (n = 8) being 50 mo (4 HAMA-negative patients were not included in this analysis due to death < 4 mo). There were no late toxicities related to 3F8 plus BG. However, one patient developed a rare peritoneal mesothelioma two years after completing therapy, attributable to prior chemo- or radiotherapy.

Correlative studies. Overall, the administration of 3F8 plus BG did not affect in vitro anti-NB immune responses, notably CR3-dependent cytotoxicity from leucocyte fractions in the presence (iC3b-ADCC) or absence (ADCC) of iC3b (p > 0.2 for all endpoints at all dose levels) or the immunophenotype of leucocytes. There was little evidence of a relationship between the dose of BG and immune endpoints. Apart from a weak association between BG dose and CD11b expression in the lymphocyte subpopulation (p = 0.002), there was no correlation between changes in the leucocyte surface receptor repertoire (CD63, CD87 and CD11a) and BG dose. In addition, there was no correlation between BG dose and CD11b expression in granulocytic and monocytic populations. In cytotoxicity assays, GM-CSF slightly decreased iC3b-ADCC, in particular in cells from patients treated with the intermediate (p < 0.01), but not the highest not the lowest BG doses. (1 → 3)β-D-glucan was

2 underwent no significant changes, while 3 manifested further increases. Six out of eight (75%) patients exhibiting PD prior to therapy had further progression after one cycle of 3F8 plus BG. There was no obvious relationship between dose and clinical effect: 4, 3, 2 and 4 patients manifested PD after receiving 10 mg/Kg/day, 20 mg/Kg/day, 40 mg/Kg/day and 80 mg/Kg/day BG, respectively. Response was, however, clearly associated with pretreatment status: 13 out of 16 patients characterized by SD at baseline responded, compared with 1 out of 8 manifesting PD at baseline (p = 0.002, Fisher’s exact test).

Survival and late toxicities. The effects of 3F8 plus BG on survival could not be evaluated in this Phase I study and all patients went on to receive subsequent therapy after coming off the study. However, all patients were monitored for late toxicities. Two out of 24 patients (29%) survived for a median duration of 71 mo after enrollment, one with residual disease. Median survival was 23 mo and median time to PD was 4 mo. Two subjects remained alive, both having responded to 3F8 plus BG. The first one (#7) had persistent skeletal MIBG uptake at a follow-up > 110 mo, receiving only 13-cis-retinoic acid after the development of HAMAs. The second one (#24) remained free of disease at a follow-up > 99 mo, having received further consolidation chemotherapy and allogeneic transplantation upon the completion of 4 cycles of 3F8 plus BG. There was no obvious effect of BG dose on progression-free survival (PFS) or overall survival (OS), and the hazard ratio (HR) for a one-level increase in dose was 1.12 (95% CI 0.73, 1.73; p = 0.6). The development of HAMAs was associated with improved OS (HR = 0.32; 95% CI 0, 9.47; p < 0.001), the median survival of patients who did not develop HAMAs (n = 12) being 25 mo and that of patients who did develop HAMAs (n = 8) being 50 mo (4 HAMA-negative patients were not included in this analysis due to death < 4 mo). There were no late toxicities related to 3F8 plus BG. However, one patient developed a rare peritoneal mesothelioma two years after completing therapy, attributable to prior chemo- or radiotherapy.

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**Figure 1.** Clinical response of neuroblastoma patients to 3F8 plus barley-derived (1 → 3), (1 → 4)-β-D-glucan. (A and B) 123I-meta-iodobenzylguanidine scans in patient #1 before (A) and after (B) one cycle of 3F8 plus barley-derived (1 → 3), (1 → 4)-β-D-glucan (BG) (administered at dose level 1, i.e., 10 mg/Kg/day).
not detected in the serum of any patient tested by the Fungitell assay (n = 5). Median absolute lymphocyte count (ALC) prior to therapy was 0.8 ± 0.5 /mL. Although ALC at enrollment did correlate with the development of HAMAs (p = 0.048), it did not correlate with OS or PFS. Ten out of 22 patients had mild elevations in circulating IL-10 on day 10 as compared with baseline conditions (mean elevation = 3.98 ± 3.17 pg/mL), whereas no patients manifested elevations in circulating IL-12. IL-10 elevations did not correlate with BG dose or therapeutic responses.

Discussion

Although many phytochemicals have purported immunomodulatory properties, the interaction between immunotherapy and these in anticancer therapy has never been investigated in formal clinical studies. Here, we report the results of the first clinical trial combining a tumor-targeting mAb with a purified immunomodulatory phytochemical. Based on our preclinical data, we chose to test the combination of the anti-NB complement-activating mAb 3F8, whose clinical effects are well characterized, with the CR3-activating glucan BG.15,16,41,42

Therapy was administered on an outpatient basis and was well tolerated even by the youngest individuals enrolled in our study. The MTD of BG was not reached and the study was completed at the planned dose of 80 mg/Kg/day. Two patients developed acute thrombocytopenia as DLT, at different BG doses. The sharp drop in the platelet count of these patients, (1) was associated with an increase in BM megakaryocytes, (2) was paralleled by the development of HAMAs and (3) was responsive to ITP therapy, suggesting an autoimmune phenomenon. This toxicity has never been previously observed with 3F8 and might have resulted from the BG-mediated activation of CR3. However, neither of these patients showed significantly higher ADCC or iC3b-ADCC post-BG. There were no other unexpected adverse events.

Objective responses were documented in 63% (15/24) of patients. All patients had previously received multiple chemotherapy regimens (median 3; range 2–6). Most patients had poor BM reserve (12/24 patients had platelet count < 100,000/μL at baseline) and would have been ineligible for most Phase I/II clinical studies involving conventional chemotherapeutics. Anti-NB responses were recorded for BM disease (histology, MIBG scans) and biochemical markers (urine catecholamines), but as reported in other mAb-based clinical trials, responses in soft tissue disease were rare. In general, responses were transient and modest. Near-to-complete resolution of extensive MBG-avid metastases after 1 cycle of 3F8 plus BG was observed in 2 chemorefractory patients. The administration of 3F8 plus BG was ineffective at inducing responses in almost all patients with PD at baseline. A dose-response correlation could not be demonstrated.

As a component of bran cereal, the nutritional value of β-glucans against cholesterol level in humans.45,46 In our study, we did not observe a cholesterol-reducing effect from any BG dose (data not shown).

The in vivo mechanism of action of β-glucans has not been adequately investigated. In a murine model, both fluorescein-labeled BG and yeast-derived (1→3),(1→6)-β-D-glucan could be detected in the spleen, lymph nodes and BM macrophages. Granulocytes with CR3-bound (1→3)-β-D-glucan-fluorescein were shown to kill iC3b-opsonized tumor cells following their recruitment to a site of complement activation.47 Since such a mechanism has not yet been demonstrated in humans, a secondary objective of our study was to investigate the immune effects of BG in patients. We observed neither a significant increase in CR3-positive neutrophils or macrophages, nor an enhancement of ADCC or iC3b-ADCC following the administration of 3F8 plus BG, nor did we detect (1→3)-β-D-glucan in the serum of 5 patients using the commercial Fungitell assay. It is unclear if these findings point to an alternative in vivo mechanism of action of BG, or to methodological issues. Yeast-derived glucan ingestion has been associated with consistent increases in IL10 mRNA levels in humans.48 However, we did not observe significant elevations in circulating IL-10 in our small patient cohort. This was probably related to the low ALC of most patients at enrollment. An intriguing finding of this study is that the development of HAMAs is associated with improved survival following the administration of 3F8 plus BG. However HAMA-positive patients continued to have refractory disease despite 3F8 plus BG and subsequent therapies. We first correlated HAMAs with increased survival in patients treated with 15,19,3F8 and hypothesized that a 3F8-induced idiotype network contributed to long-term disease control, HAMA being surrogate evidence for the activation of such a network. An idiotype network was demonstrated in a subsequent group of patients treated with 3F8 after chemotherapy on the Memorial Sloan-Kettering Cancer Center (MSKCC) N6 protocol,14 and more recently among patients treated with 3F8 + GM-CSF.7 Although survival was not the primary endpoint of our study, the correlation of OS with HAMAs suggests that an idiotype network is an important contributor to the survival of NB patients treated with 3F8-based immunotherapy. However, this observation should be tempered by the small number of patients enrolled in our trials, and by the fact that all patients who survived received further anti-NB therapy after completing all cycles of 3F8 plus BG.

mAbs nowadays constitute an established approach to cancer therapy. Yet, there is substantial room for improvements. Antitumor ADCC is Fc-dependent, but CR3-mediated mechanisms also appear to be critical.12,50–52 By activating CR3, β-glucans have been shown to enhance the clinical activity of mAbs in preclinical studies. Natural autoantibodies to a number of self antigens circulate in humans.53,54 Specifically, natural IgM responses to human NB-associated antigens are common among healthy volunteers, but absent or poor among NB patients.55 The existence of such natural antibodies may offer us a unique opportunity to exploit plant carbohydrates like β-glucans against cancer. However, as observed in the two patients who developed immune thrombocytopenia, phytochemicals have the potential to elicit autoimmune reactions.57,58 While β-glucans are not
used by oncologists, β-glucan-containing natural products such as maitake and barley are often consumed by cancer patients. Hence, the role of the patient’s diet and/or the alternative therapies to which he/she is subjected must be carefully considered when evaluating the results of immunotherapy.

We have shown that the combination of 3F8 and BG is safe. The encouraging responses observed in a heavily pretreated population support further (Phase II) studies of BG combined to other immunomodulatory agents for the therapy of NB and other tumors amenable to CR3-mediated immunotherapy. Given the low toxicity of BG and the absence of any evidence of dose-response correlations, we recommend a dose of 40–80 mg/Kg/day for future trials.

**Patients and Methods**

**Patient selection.** Patients with high-risk NB (Stage 4 disease diagnosed at > 18 mo of age or MYCN amplification plus ≥ Stage 3 tumor at any age), and a history of PD or chemoresistance were eligible. The presence of evaluable (microscopic BM metastases, elevated tumor markers, abnormal scintigraphic studies) or measurable (by CT or MRI) NB ≥ 4 weeks after completion of systemic therapy was required for eligibility. Patients with life-threatening infections or > grade 2 toxicity according to the National Cancer Institute’s Common Toxicity Criteria version 2.0 (CTC v2.0) were excluded. Conversely, patients with the following grade 3 toxicities (all clearly related to previous therapy) were included: hearing loss, fatigue, alopecia, anorexia, nausea, constipation, elevated liver function tests (LFTs) due to total parenteral nutrition (TPN) and hypomagnesemia.

**Study design.** The protocol was approved by the institutional review board of the MSKCC. Written informed consent was obtained from all patients or their guardians. One cycle consisted of oral BG (available as investigational new drug, preclinical review board of the MSKCC. Written informed consent for future trials.

**Correlative immune studies.** Patients were monitored for leukocyte priming by BG on days 1, 8, 12 and 15 of cycles 1 and 2 using a %Cr release assay. Briefly, LAN-1 and NMB7 NB cells labeled with %Cr at 100 μCi/10⁶ cells were used as targets. Leukocytes were extracted from peripheral blood samples and studied for 3F8-independent and 3F8-dependent cell-mediated cytotoxicity among granulocytes and lymphocyte cell fractions. Target cells were opsonized with iC3b using normal human serum complement. iC3b opsonized cells were then used to assay for CR3-dependent cytotoxicity in leucocyte fractions in the presence (iC3b-ADCC) or absence (ADCC) of iC3b. Sargramostim (Berlex Oncology), and IL-2 (Novartis) were employed in granulocyte (iC3b-ADCC/GM-CSF) and lymphocyte cytotoxicity assays, respectively. Plates were centrifuged at 200 × g for 4 min at 20°C, and incubated at 37°C for 4 h. Supernatants were harvested and harvested frames (Skatron). %Cr released in the supernatant was assayed using a universal γ counter. Percentage of specific release was calculated using the Equation 100% × (experimental cpm – background cpm)/(10% sodium dodecyl sulfate (SDS)-releasable cpm – background cpm), where cpm are counts per minute of released %Cr. Total release was assessed by cell lysis with 10% SDS (Sigma-Aldrich) and background release was measured in the absence of cells. The background was < 20% of total for all cell lines. Lytic units were calculated as previously described. Whole blood cells were analyzed for the expression of CD11b, CD63, CD87 and CD11a by flow cytometry using specific immunofluorescent antibodies (Becton-Dickinson) before the administration of 3F8 plus BG and on days 4, 8 and 12 of cycle 1, following previously described methods.

Serum levels of IL-10 and IL-12 were measured prior to BG and 10 d later by QuantiKine enzyme-linked immunosassays (R&D Systems). Serum collected from day 8 of cycle 1 on from patients receiving the highest BG dose was tested for the presence of (1 → 3)-β-D-glucan by the commercially available Fungitell.
modified Limulus Amebocyte Lysate assay (Associates of Cape Cod Inc.).

Statistical methods. The primary objective of the study was to determine a dosage of BG to take forward to Phase II testing. Since BG is a naturally occurring substance, we expected its toxicity to be low. We therefore required criteria other than toxicity to determine optimal dose, in the event that a MTD might not be reached. Accordingly, we analyzed immune system-, survival- and tumor response-related endpoints. The association between BG dose and immune function was explored using a general estimating equations approach, with day of treatment as co-variate and dose level as predictor. Results from immune assays were entered as continuous variables. As the relationship between dose and response may be non-monotonic, a quadratic term for dose was added to the model. Given the large number of immune endpoints, we used p < 0.005 as the cut-off for further data analysis. For the OS and PFS, logistic and Cox models, were used, respectively, with both the linear and quadratic term for dose. In a separate analysis, we explored a possible association between the development of HAMAs and OS using a landmark analysis. We used a landmark of four months, corresponding to a time point when patients would have been expected to have completed four cycles of 3F8 plus BG therapy. Patients who died before the landmark were thus excluded from the analysis. All analyses were conducted using Stata 9.2 (Stata Corp.).

Disclosure of Potential Conflicts of Interest

N-K.V.C. is the inventor of the use of glucan to enhance antibody therapy for cancer.

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