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Bortezomib Reduces Pre-Existing Antibodies to Recombinant Immunotoxins in Mice

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Recombinant immunotoxin (RIT) therapy is limited in patients by neutralizing Ab responses. Ninety percent of patients with normal immune systems make neutralizing Abs after one cycle of RIT, preventing repeated dosing. Furthermore, some patients have pre-existing Abs from environmental exposure to Pseudomonas exotoxin, the component of the RIT that elicits the neutralizing Ab response. Bortezomib is an U.S. Food and Drug Administration–approved proteasome inhibitor that selectively targets and kills plasma cells that are necessary for the neutralizing Ab response. We hypothesized that bortezomib may abrogate neutralizing Ab levels, making dosing of RIT possible in mice already immune to RIT. We immunized BALB/c mice with multiple doses of SS1P, a RIT whose Ab portion targets mesothelin. Mice with elevated Ab levels were separated into groups to receive saline, bortezomib, the pentostatin/cyclophosphamide (PC) regimen, or the bortezomib/PC (BPC) combination regimen. Four weeks after finishing therapy, plasma Ab levels were assayed, and bone marrow was harvested. The bortezomib and PC regimens significantly reduced Ab levels, and we observed fewer plasma cells in the bone marrow of bortezomib-treated mice but not in PC-treated mice. The BPC combination regimen almost completely eliminated Abs and further reduced plasma cells in the bone marrow. This regimen is more effective than individual regimens and may reduce Ab levels in patients with pre-existing neutralizing Abs to Pseudomonas exotoxin, allowing RIT treatment. The Journal of Immunology, 2015, 194: 000–000.

Forty years of rDNA technology has led to the routine use of protein therapeutics in the clinic to treat a variety of ailments. oftentimes, protein therapeutics are much more active than their small-molecule equivalents, and targeting strategies have lessened dose-limiting side effects. One limitation of protein therapeutics is the patient’s immune system recognizing exogenous proteins as foreign and forming a neutralizing Ab (NAb) response, rendering therapy ineffective or causing severe adverse clinical effects (1–4). NABs are most commonly associated with therapeutic proteins of nonhuman origin; however, “human” sequences also were shown to stimulate immune responses (1, 2, 4).

NABs are a recognized problem with therapeutic mAbs and recombinant proteins to treat cancers, autoimmune diseases, lysosomal storage diseases, hemophilia, multiple sclerosis, transplant rejection, and more (2). NABs can target epitopes on therapeutic proteins, impeding uptake, enzymatic activity, processing, or trafficking (1). Protein–Ab immune complexes are also subject to clearance from the body. Many factors contribute to the likelihood of a NAB response, including storage conditions (causing denaturation or aggregation), formulation properties, and contaminants or impurities introduced by the manufacturing process (3, 4). Not all protein therapeutics are immunogenic, and patients do not respond uniformly with NABs to those that are. The route of administration and genetic background of the patient may affect the possibility of an immune reaction, and personalized approaches to therapy may lessen the likelihood of a NAB response. Some studies showed that continuous infusion of the smallest amount of biologic necessary reduces the possibility of NABs (3). Prior exposure is also a risk factor for developing NABs (3, 5). Coadministration of immune-suppressing therapies has been studied as a means of reducing the potential for developing NABs (1, 5, 6).

The initial events that trigger the development of immune responses against protein therapeutics are not clear but are likely dependent on characteristics of the Ag and the patient. There is more evidence supporting T cell–dependent stimulation of B cells in response to protein therapeutics than T cell–independent stimulation (2). Plasma cells reside in the bone marrow or secondary lymphoid tissues and are the major Ab-producing cell type. Plasma cells are terminally differentiated B cells and can be either short- or long-lived and do not divide.

Immune suppression is an approach to prevent an immune response in a naive setting (i.e., induce tolerance) and/or reverse an ongoing immune response. Traditional immune suppressants studied to inhibit the humoral immune response include prednisone, azathioprine, rituximab, pentostatin, cyclophosphamide, methotrexate, cyclosporine A, and others. Some of these therapies completely deplete circulating B cells and can induce tolerance in naive hosts (7). Reversing an ongoing immune response is more difficult. In hosts with pre-existing humoral immune responses, these regimens are slow acting and are only partially capable of depleting NAB levels. This is because NABs are made mostly by long-lived plasma cells that do not divide; therefore, immune suppressants that target proliferating cells are ineffective (8). Furthermore, plasma cells lack common B cell markers, making them resistant to anti-CD20–targeted therapy.

The arrival of proteasome inhibitors has filled the need for a powerful and specific way to target plasma cells, a cell type that is highly dependent on proteasome activity. Cells that accumulate...
mismfolded proteins die through the unfolded protein response (9). Bortezomib is a reversible proteasome inhibitor that targets both short- and long-lived plasma cells as a result of their high rates of Ig production (10). Although bortezomib was initially approved for multiple myeloma, animal studies proved that proteasome inhibition could also kill normal plasma cells. Moreover, plasma cells were depleted as early as 48 h after treatment. Several clinical trials have studied the off-label uses of bortezomib. Some studies for the prevention of Ab-mediated rejection in renal transplant patients reported encouraging results, and there are also reports of positive outcomes in the treatment of autoimmune hemolytic anemia, rheumatoid arthritis, and systemic lupus erythematosus (8).

Recombinant immunotoxins (RITs) are recombinant chimeric protein therapeutics composed of a targeting moiety fused to a toxin. The targeting moiety is an Ab or Ab fragment, and the toxin is usually a bacterial toxin. Our group has made RITs with Ab fragments targeting CD22, mesothelin, and others. Many toxins have been evaluated for use in constructing RITs, although our group exclusively uses the toxin derived from the bacteria Pseudomonas aeruginosa. The 38-kDa fragment of Pseudomonas exotoxin A (PE38) elicits its cytotoxic effect by ribosylating eukaryotic elongation factor 2 in the host cell. Without functional elongation factor 2, target cells cannot synthesize protein, and they die (11). PE38, owing to its bacterial origin, is immunogenic in humans and results in a NAb response in patients with normal immune systems. In patients with mesothelioma who presumably have normal immune systems, RIT treatment is limited to one or two treatment cycles before Abs develop (12). Tumor responses have not been observed in these patients. In patients with hairy cell leukemia who are immunologically impaired, NAb responses to RITs are rarer, and durable complete responses are common (13). In a phase I clinical trial, only 10% (38%) of 26 evaluable hairy cell leukemia patients formed NAbs; of these, only one patient developed NABs after the first cycle (13).

Previously, we showed improved responses to RIT in mesothelioma patients by immune suppression using pentostatin and cyclophosphamide (6). This immune-suppression regimen delayed Ab formation in RIT-naive patients, allowing up to six cycles of RIT, whereas in previous studies without immune suppression, patients could receive only one or two cycles of RIT. Although other mechanisms cannot be ruled out, it is likely that the increased antitumor response was due to increased and more effective RIT delivery without neutralization. However, a subset of patients cannot receive even one cycle of RIT because of pre-existing NAbs to PE due to environmental exposure to P. aeruginosa (5). To treat this population of patients or patients who have developed NABs during the course of treatment, we investigated using bortezomib to eliminate pre-existing Abs to PE.

Murine models have been used to predict immunogenicity of clinical RITs (14–17), but suppression of immunogenicity in sensitized hosts has not been addressed. In this study, we examined mice with active immunological responses to RIT to determine the degree of immune suppression imparted by bortezomib alone and in combination with the previously reported pentostatin/cyclophosphamide (PC) regimen. Bortezomib and PC affect plasma cells and T/B cells, respectively, so we hypothesized that immune suppression in combination treatment would be additive or greater. To assess immune suppression we used immune complex captured (ICC)-ELISA specific for anti-RIT–binding Abs. Previous studies demonstrated that binding Ab levels determined by ELISA correlated with NAB levels (13). Our goal is to develop a combination regimen of U.S. Food and Drug Administration (FDA)-approved therapies to abrogate pre-existing Abs to PE, permitting the use of RITs in patients who otherwise could not receive therapy.

### Materials and Methods

**Mice and reagents**

Female BALB/c mice (8–10 wk old) were purchased from Frederick Cancer Research Facility (Frederick, MD). Mice were treated according to an approved animal protocol and maintained in a pathogen-free facility. Mice were immunized weekly with the RIT SS1P (5 μg/mouse, i.v.; Advanced BioScience Laboratories). Bortezomib (1 mg/kg; Selleck Chemicals) was injected i.v., and cyclophosphamide (50 mg/kg; Baxter) and pentostatin (1 mg/kg; Hospira) were injected i.p., according to the schedules shown in Table I. Control mice received saline injections.

**ELISAs to determine Ab level**

Blood was drawn into heparinized capillary tubes before being transferred to microcentrifuge tubes. Samples were centrifuged, and plasma supernatants were transferred to a new tube and kept at −80°C until analyzed. ICC-ELISAs were performed as previously described (18). Briefly, high-affinity binding plates were coated with 50 ng mesothelin-Fc protein (19). In another tube, plasma was mixed with SS1P (1 μg/ml) and incubated overnight at 4°C. Plates were blocked with blocking buffer, washed with PBS, and incubated with plasma/SS1P mixture. After washing, anti-SS1P Abs were detected by goat anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories) and tetramethylbenzidine (TMB substrate kit; Thermo Scientific). Each plate contained triplicates of 2-fold dilutions of the anti-PE38 mAb, IP37, as a binding standard (18). Each sample was serially diluted, and reactivity was measured at eight dilution points. Standard curves and each unknown plasma concentration curve were fit by a four-parameter logistic curve model using GraphPad Prism (GraphPad). Plasma Ab concentrations were expressed relative to IP37 concentration.

**Plasma cell detection**

Four weeks after finishing therapy, mice were euthanized and bone marrow was harvested immediately. Single-cell suspensions were made by successive passes through a fine-gauge needle. RBCs were lysed by suspension in ACK lysis buffer (Lonza). Cells were incubated with anti-CD16/CD32 (FcBlock; BD Biosciences) before incubation with anti-CD138-PE (BioLegend), anti–CD38-FITC (BioLegend), anti–CD19-BV421 (BioLegend), and anti-B220/CD45R—allphycocyanin (BD Biosciences) and analyzed on a LSRFortessa flow cytometer (BD Biosciences). At least 250,000 live events were acquired per sample. Data analysis was performed with FlowJo (TreeStar).

**Statistics**

All statistical tests were performed with GraphPad Prism (GraphPad). The Student two-tailed t test was used to analyze plasma cell depletion. The Mann–Whitney nonparametric test was used for comparisons between experimental groups in the immunogenicity study. The p values < 0.05

### Table I. Dosing schedules for mice receiving bortezomib, PC, and BPC regimens

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were considered statistically significant. The entire animal experiment was
done in duplicate, and similar results were obtained.

Results
NAbs are a major impediment to the clinical translation of therapeu-
tic proteins. About 5% of patients who could benefit from
RIT therapy cannot be treated because of pre-existing Abs from
environmental exposure to *P. aeruginosa*. In addition, we have

discussed a growing cohort of patients who can no longer receive RIT
therapy as a result of formation of NAb during treatment. Our
goal was to develop and characterize a universal immunosup-
pression regimen that would permit the use of therapeutic proteins
in sensitized patients. We tested three immunosuppressive regi-
mens in immunized mice to ascertain the degree of Ab depletion.
We chose therapies that would target Ab-producing plasma cells,
as well as effector B and T cells. Bortezomib was initially ap-

![Graphs A, B, and C showing the results of the immunosuppressive regimens.](http://www.jimmunol.org/dx.doi/10.1172/JCI85346)

**FIGURE 1.** Bortezomib (A), PC (B), and BPC (C) regimens reduce anti-SS1P Ab levels. Plasma was collected 4 wk after therapy, and anti-SS1P Ab
levels were determined by ELISA. Results for individual mice and group averages are shown. Initial Ab levels for each mouse were normalized to 100%,
and Ab levels after treatment were scaled relative to initial levels. ELISAs were performed in triplicate. Error bars indicate SEM. The entire experiment was
repeated once.
proved for multiple myeloma, but it was later demonstrated to kill nonneoplastic plasma cells as well, because of their high rates of protein synthesis (20). Activated B cells are also targeted by bortezomib, decreasing both Ig production and proliferation, and even causing apoptosis (21). Because of bortezomib’s broad targeting of Ab-producing cells, we first evaluated whether bortezomib alone could abrogate pre-existing Abs in previously immunized mice. To achieve a robust humoral immune response we injected mice five times with SS1P, a mesothelin-targeted RIT bearing PE38, which is immunogenic in BALB/c mice. Ab levels were determined by ICC-ELISA, as previously described (18). ICC-ELISA results correlate with neutralization assays because ICC-ELISA detects Ab that reacts with the native form of Ag. We showed previously that binding Ab levels correlate with NAb levels (13). After initial Ab levels were determined, we treated mice with bortezomib (1 mg/kg body weight, dosed i.v.) twice a week for 5.5 wk (Table I). A pilot experiment indicated that we could safely dose mice with 1.5 mg/kg bortezomib (Supplemental Fig. 1), but we decided to use the lower amount because this dose and schedule are comparable to the recommended twice weekly dosing of bortezomib (1.3 mg/m², dosed i.v. or s.c.) for the initial treatment of multiple myeloma (22). The half-lives of serum IgGs in mice have been determined, and IgG isotypes have the longest half-lives at 6–8 d (23). Because of this, we waited 4 wk after finishing the bortezomib regimen to allow the natural elimination of circulating Ig before determining the degree of Ab reduction. Bortezomib treatment reduced anti-SS1P Abs from an average of 138 μg/ml to an average of 44 μg/ml (p, 0.01) (Figs. 1, 2). Saline treatment did not reduce the level of anti-SS1P Abs in control mice; mean anti-SS1P Ab levels before and after saline treatment were 120 and 102 μg/ml, respectively (p = 0.77).

Although bortezomib alone induced a striking decrease in the levels of pre-existing Abs, our goal was to drive levels as low as possible. Previously, we described immune depletion with pentostatin and cyclophosphamide in naive mice that safely abrogated the ability to form anti-SS1P Abs (7). In our subsequent experiment, we chose to incorporate bortezomib into the previously established PC regimen. We used pentostatin at 1 mg/kg and cyclophosphamide at 50 mg/kg, which translates to ~3 and 240 mg/m², respectively. Both of these are within their recommended clinical ranges (24). The PC regimen consists of an initial induction period followed by a longer and less intensive maintenance period. We timed the PC regimen to conclude simultaneously with the bortezomib regimen. We determined the degree of Ab reduction 4 wk after the end of therapy. Bortezomib/PC (BPC) treatment reduced anti-SS1P Abs from an average of 67 μg/ml to an average of 8 μg/ml (p < 0.0001) (Figs. 1, 2). Some weight loss was associated with this combination regimen, but no deaths occurred. We set up a third experiment to control for the effect of PC in the absence of bortezomib, using the same PC dosing and scheduling parameters as in the BPC experiment. PC treatment reduced anti-SS1P Abs from an average of 82 μg/ml to an average of 42 μg/ml (p < 0.01) (Figs. 1, 2).

Plasma cells are terminally differentiated B cells and the major Ab-producing cell type; thus, they are the most pertinent to our study. Common B cell markers, including CD20 and B220/CD45R, are lost during the differentiation process, and the plasma cell markers CD38 and CD138 are gained (25). Using these markers,

![FIGURE 2.](http://www.jimmunol.org/)

The bortezomib and PC regimens reduce anti-SS1P Ab levels, and the BPC regimen almost completely eliminates them. Each regimen decreased Ab levels relative to initial levels (percentage of anti-SS1P IgG reduction and p values are relative to saline in each experiment and indicated on respective graphs). The horizontal lines represent the median. There was no difference in initial Ab levels among the bortezomib, PC, and BPC groups. After treatment, the BPC regimen outperformed the bortezomib and PC regimens (both p < 0.0001). Data shown are a compilation from two independent experiments.
we examined plasma cells [as defined previously (26); CD19+, B220/CD45R, CD38, and CD138]. Expression was analyzed by flow cytometry. (A) Dot plots representative of experimental groups; CD38+/CD138+ plasma cells are gated in the rectangle, and percentage of total bone marrow cells is indicated above the gated cells. (B) Percentage of plasma cells detected in the bone marrow of control and experimental mice. The experiment was repeated on separate days with similar results, and data were combined. Error bars indicate SEM. *p < 0.05, **p < 0.001 versus control (saline).

we examined plasma cells [as defined previously (26); CD19+, B220/CD45R, CD38high, CD138high] in the bone marrow of experimental and control mice to understand the mechanism of immune suppression (Supplemental Fig. 2). Plasma cells accounted for 0.43% of total mononucleated bone marrow cells in control mice (Fig. 3). This observation is consistent with the reported abundance of plasma cells in the bone marrow of mice (27) and humans (28). In mice treated with the bortezomib regimen, plasma cells were reduced to 0.20% of total mononucleated bone marrow cells, a >50% reduction compared with control mice. In mice treated with the BPC regimen we observed a further reduction in plasma cells to just 0.14% of total mononucleated bone marrow cells, a nearly 70% reduction compared with control mice. We observed slightly fewer plasma cells in the bone marrow of mice treated with the PC regimen compared with control mice, but this finding was not statistically significant (0.35 and 0.43%, respectively; p = 0.102).

To determine whether the efficacy of bortezomib is independent of how recent immunity was acquired, we tested the bortezomib regimen on immunized mice after a resting period. Immunized mice were rested for 9 mo before treatment with the bortezomib regimen (Table I). More Ab was detected after this resting period (p < 0.05) (Fig. 4). It is unclear whether Ab levels increased or their affinity had increased, because a limitation of our ELISA is that the reactivity of polyclonal Ab was converted to the amount of standard mAb. Subsequent bortezomib treatment lowered Ab levels relative to those before treatment (p < 0.05) but not to levels prior to the 9-mo resting period (p = 0.41).

Discussion
RITs are effective when delivered in sufficient quantity, but their use is limited by the induction of NAbs. Previously, we described a regimen to slow the induction of NAbs in naive patients. This immune-suppression regimen allowed up to six cycles of RIT in mesothelioma patients, and better clinical responses were observed. However, a subset of patients cannot receive even one cycle of RIT or are taken off therapy because of pre-existing or development of NAbs to P. aeruginosa. In our current study we...
investigated the use of bortezomib in immune-suppression regimens to eliminate pre-existing Abs to PE. Bortezomib has been used similarly to eliminate pre-existing Abs (29). In children receiving enzyme-replacement therapy to manage Pompe disease, bortezomib was used in combination with cyclophosphamide, rituximab, methotrexate, and i.v. Ig. This salvage therapy successfully depleted Abs, allowing continued enzyme-replacement therapy; however, this regimen could not be safely translated to adult mesothelioma patients.

Bortezomib broadly targets Ab-producing cells; therefore, in our first experiment we investigated whether bortezomib alone could induce tolerance in previously immunized mice. With our future goal of clinical translation in mind, we chose a dose and schedule of bortezomib similar to what can be safely achieved in patients clinically. Bortezomib was well tolerated, and no weight loss was observed. The single-agent bortezomib regimen reduced anti-SS1P IgG levels by an average of 68% compared with an average reduction of 15% in control mice. We hypothesized that incorporation of bortezomib into our previously characterized PC regimen would have at least an additive effect because of the different ways in which these regimens act. The BPC combination regimen reduced anti-SS1P IgG levels by an average of 88%. Some weight loss was associated with this combination regimen, but all mice survived therapy. In our third experiment we determined the effect of PC in the absence of bortezomib and observed a 49% reduction in anti-SS1P IgG levels. The PC regimen was well tolerated, and no weight loss was observed. Previous studies demonstrated that binding Ab levels determined by ICC-ELISA correlated with NAb levels (13).

The reduction in plasma cells observed in the bortezomib and BPC experiments is consistent with the known mechanism of action of bortezomib. The PC regimen did not significantly affect plasma cells, as expected. Plasma cells are long-lived; thus, they are directly resistant to antiproliferative therapy. The slight reduction in plasma cells may be an indirect effect of the PC regimen, because fewer B cells would be present to mature into plasma cells. However, the slight reduction in plasma cells cannot explain the 49% reduction in anti-SS1P Abs. Our results suggest that some anti-SS1P Ab is being made by Ab-producing mature B cells or some other cell type that is sensitive to the PC regimen.

The BPC combination regimen is most effective at suppressing pre-existing SS1P Abs, reducing Ab levels almost 90%. Successful clinical translation of these findings will depend on a variety of factors. In this animal model, mice received repeated injections of the immunogen SS1P. This immune-response mechanism may differ between mice and humans; however, the effect of bortezomib on function may very well be the same. The efficacy of bortezomib is independent of how recently immunity was acquired, because mice respond similarly with or without a 9-mo resting period. It is unknown whether any of the regimens induce long-term tolerance, and the clinical implications of such tolerance are also unknown. We plan to conduct additional experiments to measure secondary immune responses in treated and control mice. Some weight loss was observed as a result of the BPC regimen, which was not observed with either the bortezomib or PC regimen. Successful clinical translation may require modifications to dose or scheduling. In humans, peripheral neuropathy is a significant toxicity of bortezomib, requiring dose modification and potential changes in treatment plans (30). Carfilzomib, also a 20S proteasome inhibitor, is seen as a successor to bortezomib because of its reduced risk for toxicity, particularly peripheral neuropathy (31). Because of the similar mechanisms of action, carfilzomib may be able to replace bortezomib in our regimen with less risk for toxicity. Recently, we found that tofacitinib suppressed Ab responses to RIT by blocking B cell development and impairing the formation of germinal centers (32). Because tofacitinib is now approved by the FDA, it could be used as combination therapy with bortezomib instead of the PC regimen for B cell control with fewer side effects. Our group is also heavily invested in deimmunizing PE via the identification and removal of B and T cell epitopes. Deimmunization circumvents the need for immune-tolerance regimens; however, it requires restarting the regulatory-approval process. In contrast, developing regimens from FDA-approved drugs provides a much faster way of meeting the needs of patients. Furthermore, it is important to note that the immune-tolerance regimens that we investigated are not specific for PE and may be applicable to other immunogenic therapies as well.

Disclosures
The authors have no financial conflicts of interest.

References
5. Wayne, A. S., D. J. Fitzgerald, R. J. Kreitman, and I. Pastan. 2014. Immuno-
14. Liu, W., M. Onda, C. Kim, L. Xiang, J. E. Weldon, B. Lee, and I. Pastan. 2012. A recombinant immunotoxin engineered for increased stability by adding a disul-


Bortezomib Reduces Preexisting Antibodies to Recombinant Immunotoxins in Mice

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SUPPORTING FIGURES
Figure S1: The bortezomib regimen (1 mg/kg) is safe in mice. Dose escalation experiment was performed in naïve mice. Mice (n=4 each group) were injected i.v. twice weekly with bortezomib dissolved in saline. Animal health was inferred by body mass over the 5.5 week period. Mice in the 1 and 1.5 mg/kg groups appeared similar as control (saline) mice. Mice in the 2 and 2.5 mg/kg groups lost significant weight and were sacrificed in week 2. Experiment was performed once. Error bars indicate SEM.
Figure S2: Gating strategy to detect plasma cells (defined as CD19 and B220/CD45R double-negative, CD38 and CD138 double-positive). Debris (panel A) and doublets (panels B and C) were first gated out. CD19 and B220/CD45R double-negative cells were selected (panel D). CD38 and CD138 double-positive cells were then selected as plasma cells (panel E).