Brief report

Alpha phenyl-tert-butyl nitrone (PBN) protects syngeneic marrow transplant recipients from the lethal cytokine syndrome occurring after agonistic CD40 antibody administration

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Administration of agonistic monoclonal antibodies or recombinant cytokines is a potential approach to enhance antitumor immunity in bone marrow (BM) transplant recipients, but is complicated by toxicity resulting from proinflammatory cytokine-mediated vital organ damage. We used a murine syngeneic bone marrow transplant (BMT) model, in which administration of anti-CD40 antibody early after BMT results in overproduction of interleukin-12 (IL-12) and interferon-γ (IFN-γ), and lethal gut toxicity to examine the protective effect of the spin trap inhibitor, alpha phenyl-tert-butyl nitrone (PBN). Administration of PBN protected transplant recipients from mortality by significantly attenuating gut toxicity, but did not effect a reduction in the levels of proinflammatory cytokines (IL-12, IFN-γ), tumor necrosis factor α (TNF-α), or nitrate/nitrite. Moreover, PBN did not compromise anti-CD40 antibody–mediated antitumor effects in a nontransplantation lymphoma model. Collectively, these data suggest that PBN administration may represent a novel approach for reduction of toxicity without compromise of antitumor effects resulting from administration of therapeutic antibodies in both transplantation and nontransplantation settings. (Blood. 2005;105:428-431)

Introduction

Disease recurrence is a major cause of treatment failure after bone marrow transplantation (BMT), particularly in the autologous transplantation setting. Adjunctive immunotherapeutic approaches in the peritransplantation period offer the potential to further reduce the risk of relapse. The administration of recombinant cytokines (eg, interleukin-2 [IL-2], IL-12, IL-15, IL-18)1-4 or agonistic monoclonal antibodies (eg, anti-CD40)5 6 that can activate cells of the innate and adaptive immune system have been shown to be an effective therapeutic approach for inducing antitumor immunity. The use of these agents, however, is often complicated by systemic toxicity that can be life threatening and necessitate dose reduction or premature cessation of therapy.1,2,7-9 When administered in the setting of a transplantation conditioning regimen that also induces an inflammatory milieu,10 these agents may further exacerbate toxicity.

Alpha phenyl-tert-butyl nitrone (PBN) is a spin trap agent that is able to react with and trap free radicals yielding a characteristic spectrum that can be visualized by electron paramagnetic resonance spectroscopy.11 PBN reacts not only with oxygen radicals, but also with carbon- and nitrogen-based radicals, making it an attractive agent for protection against oxidative damage in a number of murine models characterized by overproduction of proinflammatory cytokines.12-14 In this study, we used a murine syngeneic BMT model in which administration of agonistic CD40 antibody to mice early after transplantation results in lethal gut toxicity due to production of high levels of IL-12 and interferon-γ (IFN-γ)15-16 to determine if PBN could protect mice from this lethal cytokine syndrome. We also examined whether PBN compromised antitumor effects mediated by agonistic CD40 antibody in a murine lymphoma model.

Study design

Mice

C57BL/6 (B6) (H-2b) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). All animals were housed in the American Association for Laboratory Animal Care (AALAC)–accredited Animal Resource Center of the Medical College of Wisconsin (MCW).

Reagents

Antimouse CD40 antibody (FGK115, rat immunoglobulin G1 [IgG1]) was produced as ascites and diluted in phosphate-buffered saline (PBS) for injections as previously described.15 Measurement of endotoxin in the antibody preparation using the limulus amebocyte assay was 15.5 EU/mg IgG protein. PBN was obtained from Sigma (St Louis, MO) and dissolved...
in dimethylsulfoxide (DMSO) prior to administration. Rat IgG was obtained from Sigma.

**Bone marrow transplantation**

Bone marrow (BM) was flushed from donor femurs and tibias, passed through sterile mesh filters, and resuspended in Dulbecco modified media prior to injection. Host mice were conditioned with lethal total body irradiation (TBI; 1000 cGy) administered as a single exposure at a dose rate of 67 cGy using a Shepherd Mark I Cesium Irradiator (J. L. Shepherd and Associates, San Fernando, CA). Irradiated recipients received a single intravenous injection of 5 × 10^6 BM cells within 4 hours of irradiation.

**Histologic analysis**

Representative samples of lung, liver, small intestine, and colon were obtained from transplant recipients and fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin, cut into 5-micrometer-thick sections, and stained with hematoxylin and eosin (H and E). No abnormalities were observed in the lungs, small intestines, or livers of anti-CD40 antibody-treated animals. A semiquantitative scoring system was used to account for the extent of crypt cell apoptosis and destruction in the colon (0 for normal, 1 for mild, 2 for moderate, and 3 for severe for each of these 2 parameters; maximal score, 6). Scores were added to provide a composite score for each animal. All slides were coded and read in a blinded fashion.

**ELISA assays**

Sera were obtained from transplant recipients by tail vein or retro-orbital bleeds and assayed for tumor necrosis factor α (TNF-α), IFN-γ, and IL-12 levels in standard enzyme-linked immunosorbent assays (ELISAs; Pharmingen, San Diego, CA). Plasma obtained from transplant recipients was passed through an Amicon 30-kDa molecular weight cut-off filter (Millipore, Bedford, MA) and then assayed for total nitrate/nitrite concentration (Cayman Chemical, Ann Arbor, MI).

**Tumor model**

EL4 (H-2^k) T-cell lymphoma cells that do not express CD40 were used as a source of tumor. B6 mice were administered 10^7 EL4 cells intravenously and then treated with either anti-CD40 or rat IgG control antibody (500 µg/d) on days 3 to 6, as previously described by Tutt et al.17

**Statistics**

For comparison of cytokine and nitrate/nitrite levels between respective groups, the Friedman nonparametric test for randomized block design was used with each block representing an individual experiment. Histopathologic scores among groups were compared using the Mann Whitney U test. Survival curves were constructed using the Kaplan-Meier product limit estimator and compared using the log-rank rest. P less than or equal to .05 was deemed to be significant in all experiments.

**Results and discussion**

Studies were performed to determine whether PBN could attenuate lethality induced by anti-CD40 antibody administration early after transplantation. Lethally irradiated B6 mice received transplants of B6 BM cells and were then given either anti-CD40 (8 µg/d) or rat IgG (10 µg/d) antibody on days 1 to 4 after transplantation. Mice given rat IgG were treated with PBN (IgG/PBN), while animals treated with anti-CD40 were given either DMSO (CD40/DMSO) or PBN (CD40/PBN). PBN was administered as a loading dose of 300 mg/kg intraperitoneally beginning on day one after transplantation. Mice treated with anti-CD40 were administered either PBN (n = 16) or DMSO (n = 16) twice daily for 6 days beginning on the day of BMT. Actual survival is depicted. Data are cumulative results from 2 independent experiments. For comparison of cytokine and nitrate/nitrite levels between respective groups, the Friedman nonparametric test for randomized block design was used. Histopathologic scores among groups were compared using the Mann Whitney U test. Survival curves were constructed using the Kaplan-Meier product limit estimator and compared using the log-rank rest. P less than or equal to .05 was deemed to be significant in all experiments.
30 minutes prior to TBI followed by a dose of 50 mg/kg 3 to 6 hours after BMT and then twice daily for the next 5 days. The loading dose was based on studies demonstrating that this dose provided maximal protection in mice in a lipopolysaccharide (LPS)–induced shock model. All CD40/DMSO-treated animals died within 8 days of transplantation (Figure 1A). In contrast, CD40/PBN-treated mice had significantly superior survival and recovered their pretransplantation weights within 2 weeks after BMT (Figure 1B). Histologic studies revealed that the colon was the primary site of organ damage and that CD40/PBN-treated mice had significantly less pathologic damage than CD40/DMSO-treated animals (mean score, 2.7 ± 0.5 versus 4.9 ± 0.5; P = .014) (Figure 1C). Representative colonic tissue demonstrated diffuse crypt destruction and cellular necrosis in CD40/DMSO-treated mice (Figure 1E) when compared with control animals (Figure 1D). Administration of PBN, however, attenuated the observed histologic damage (Figure 1F).

PBN has been shown to be protective in a number of murine models such as dextran-induced colitis, chemically induced diabetes mellitus, and endotoxin-mediated shock. Proposed mechanisms for the protective effect of PBN have included down-regulation of TNF, IFN-γ, and nitric oxide; increased production of IL-10; and inhibition of free radical production. Cytokines, such as IL-12 and IFN-γ, are important downstream mediators of the antitumor effects induced by agonistic CD40 antibody administration. Therefore, cytokine and nitrate/nitrite levels were measured to explore the mechanism by which mice were protected from mortality and to determine whether PBN inhibited cytokines that are also important in mediating antitumor immunity. As previously described, administration of anti-CD40 antibody resulted in a significant increase in IL-12 and IFN-γ levels compared with IgG/PBN-treated mice (Figure 2A-B). There was, however, no significant difference in either IL-12, IFN-γ, TNF-α, or nitrate/nitrite levels in CD40/PBN-treated compared with CD40/DMSO-treated animals (Figure 2). Serum IL-10 levels were also undetectable in both CD40/DMSO-treated and CD40/PBN-treated mice (data not shown), indicating that increased IL-10 production was not a relevant mechanism in this model. We believe that this protective effect was most likely attributable to a reduction in free radical–mediated tissue damage, which is consistent with the known properties of PBN. A putative role for oxidative stress as a cause of toxicity after treatment with recombinant cytokines or agonistic antibodies is supported by studies showing that lung injury occurring after IL-2 therapy is mediated by oxygen-derived free radicals and that administration of a superoxide dismutase mimetic blocks IL-2–mediated hypotension, thereby allowing for IL-2 dose escalation and an improved therapeutic response.

To directly test whether PBN compromised the antitumor effects of agonistic CD40 antibody, B6 mice were intravenously administered 10⁷ EL4 cells and then treated on days 3 to 6 with either rat IgG or anti-CD40 antibody (500 µg/d). Since EL4 cells do not express CD40, tumor regression mediated by anti-CD40 antibody is attributable to augmentation of host immunity and has been shown to be dependent upon IFN-γ. Mice given rat IgG were treated with DMSO, while animals treated with anti-CD40 were given either DMSO or PBN (50 mg/kg) twice daily on days 3 to 6. CD40/DMSO-treated mice had significantly prolonged survival when compared with IgG/DMSO control animals (P = .003) (Figure 3). PBN did not compromise the antitumor effects mediated by agonistic CD40 antibody, as CD40/PBN-treated animals had similar survival to CD40/DMSO mice. In this model, however, no lethality occurs due to anti-CD40 antibody administration. Therefore, whether PBN compromises antitumor effects in mice that receive TBI and then are administered agonistic CD40 antibody to induce toxicity is not resolved by these studies. Experiments are currently under way to address this issue.

In conclusion, use of agents, such as PBN, may be an effective approach for dissociation of beneficial immune enhancing from deleterious side effects resulting from administration of therapeutic antibodies or recombinant cytokines in BMT or other nontransplantation settings.
References


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