Brief report

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

Maria Gendelman, Nadine Halligan, Richard Komorowski, Brent Logan, William J. Murphy, Bruce R. Blazar, Kirkwood A. Pritchard Jr, and William R. Drobyski

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 due to 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 affect 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)

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 (AAALAC)–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. 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

From the Bone Marrow Transplant Program and the Departments of Medicine, Pathology, Biostatistics, and Pediatric Surgery, Medical College of Wisconsin, Milwaukee, WI; the Department of Microbiology and Immunology, University of Nevada–Reno, Reno, NV; and the Department of Pediatrics, University of Minnesota, Minneapolis, MN.


Supported by grants from the National Institutes of Health (HL64603, HL55388, HL63452, and CA95572) and the Midwest Athletes Against Childhood Cancer Fund (Milwaukee, WI).

Reprints: William R. Drobyski, Bone Marrow Transplant Program, 9200 W Wisconsin Ave, Milwaukee, WI 53226; e-mail: bill@bmt.mcw.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.
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 × 10⁶ 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-2b) T-cell lymphoma cells that do not express CD40 were used as a source of tumor. B6 mice were administered 10⁷ 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...
PBN, however, attenuated the observed histologic damage (Figure 1F) when compared with control animals (Figure 1D). Administration of 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 mimic 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 lymphoma cells and then treated with either rat IgG/DMSO or anti-CD40 for 4 days beginning on day 3 after EL4 administration. Mice treated with anti-CD40 were administered either DMSO or PBN (6-11/group) for 5 days (days 0-4) after BMT. Mice were bled on days beginning on day one after transplantation. Mice treated with anti-CD40 were administered 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 (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.
References


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

Maria Gendelman, Nadine Halligan, Richard Komorowski, Brent Logan, William J. Murphy, Bruce R. Blazar, Kirkwood A. Pritchard, Jr and William R. Drobyski