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Endogenous Nitric Oxide Protects against T Cell-Dependent Lethality during Graft-versus-Host Disease and Idiopathic Pneumonia Syndrome

David Hongo,* J. Scott Bryson,† Alan M. Kaplan,* and Donald A. Cohen2*

The pathogenesis of idiopathic pneumonia syndrome (IPS), a noninfectious pulmonary complication of allogeneic bone marrow transplantation (BMT), has not been fully elucidated. However, several contributing factors have been proposed, including lung injury caused by reactive oxygen and nitrogen intermediates during preconditioning and development of graft-vs-host disease (GVHD). Studies on the role of reactive oxygen and nitrogen intermediates in IPS have yielded conflicting results. We have described a murine model of IPS, in which the onset of lung inflammation was delayed by several weeks relative to GVHD. This study evaluated whether the delay in onset of IPS was due to slow turnover of NO-producing, immunosuppressive alveolar macrophages (AM) following BMT. The results indicated that AM were immunosuppressive due to synthesis of NO. However, NO production and immunosuppressive activity by AM did not decline after BMT, but rather remained elevated throughout the 12-wk development of GVHD and IPS. In a 14-day model of IPS, continuous inhibition of NO with aminoguanidine (AG) reduced signs of IPS/GVHD, but also led to higher mortality. When AG treatment was initiated after onset of IPS/GVHD, rapid mortality occurred that depended on the severity of IPS/GVHD. AG-enhanced mortality was not due to inhibition of marrow engraftment, elevated serum TNF-α, liver injury, or hypertensive responses. In contrast, T cells were involved, because depletion of CD4+ lymphocytes 24 h before AG treatment prevented mortality. Thus, NO production following allogeneic BMT affords a protective effect that helps down-regulate injury caused by T cells during GVHD and IPS. The Journal of Immunology, 2004, 173: 1744–1756.

Pulmonary dysfunction and graft-vs-host disease (GVHD) remain major complications following bone marrow transplantation (BMT). Idiopathic pneumonia syndrome (IPS), a noninfectious lung complication of BMT, accounts for nearly 50% of all the cases of interstitial pneumonitis after BMT (1–3), and is associated with the development of bronchiolitis, vasculitis, and interstitial pneumonitis within 3–24 mo after transplant. The events leading to the development of IPS have not been completely elucidated, and current treatments have not reduced the high mortality rates associated with IPS. A recent study by Fukuda et al. (4) found that patients receiving myeloablative conditioning before allogeneic BMT had a significantly higher incidence of IPS than patients receiving nonmyeloablative conditioning, and that the severity of GVHD positively correlated with the frequency of IPS. These findings suggest that inflammatory tissue injury associated with myeloablative conditioning and GVHD may be predisposing factors for the development of IPS. Both oxidative and nitrosative stress are thought to play important roles in tissue injury related to GVHD and IPS (5–8). Although oxidative stress is an important byproduct of ionizing radiation and some cytotoxic drugs used in conditioning and is generated during inflammatory responses, nitrosative stress is a feature more often associated with inflammation, which occurs following myeloablative conditioning as well as during GVHD. An important role of NO generation in the development and severity of GVHD and IPS appears certain; however, whether that role is detrimental or beneficial remains controversial.

Many of the detrimental effects of NO production have been associated with high levels of NO synthesis via the inducible form of NO synthase (iNOS). Tissue injury can be mediated directly by interaction of NO with target tissues or indirectly via proinflammatory actions of NO. Ellison et al. (9) recently demonstrated that release of NO by activated macrophages in the intestines of mice with GVHD causes apoptosis in intestinal epithelial cells. NO has also been shown to kill alveolar type II epithelial cells, independently of their cell cycle status, whereas the reactive nitrogen species, NO2 or peroxynitrite, selectively kill cells that are actively cycling (10). This direct cytotoxic effect on lung epithelium has been proposed to be due to NO-mediated activation of the pro-apoptotic ERK kinase, JNK, and down-regulation of antiapoptotic NF-κB (11, 12). Injury to intestinal epithelial cells can disrupt the intestinal barrier, allowing bacterial translocation and further activation of intestinal macrophages (13). Many of the injurious effects of NO may be indirectly mediated by proinflammatory actions of reactive nitrogen intermediates (RNI) (14). NO production in the lung following bacterial exposure has also been shown to mediate expression of the chemokine, MIP-2, promoting the migration of inflammatory cells into tissues with high NO production (15).
The beneficial effects of NO production on inflammatory diseases have also been described in a variety of situations, and often have been shown to occur simultaneously with the detrimental effects and to affect many of the same processes. For example, NO and RNI can inhibit P-selectin expression by platelets and neutrophils, which can reduce their adherence to vascular endothelium (14). NO can also inhibit the activation of cyclooxygenase and the production of superoxide anion by leukocytes (14). The best described anti-inflammatory functions of NO are the ability to inhibit Ag presentation and T cell proliferation (16). The immunosuppressive activity of alveolar macrophages (AM) in normal lung is well known (17–20). NO production is primarily responsible for the immunosuppressive activity of AM in the lung, and activation of macrophages from other tissue sites also enhances their immunosuppressive activity via NO production (21). Thus, the effect of NO and RNI in inflammatory disease settings is most likely related to a balance between the detrimental and beneficial effects, which may be affected by the stage and severity of inflammatory diseases, such as occurs in GVHD and IPS.

We have described a murine model of IPS in which GVHD and lung disease were induced by an allogeneic parental-to-F1 (C57BL/6-to-DBA/2) BMT following total body irradiation (22). This experimental transplant strategy has traditionally been used to study acute GVHD, a syndrome in which CD8+ T cells have been implicated to play a major role (23, 24). In this model of IPS, lung inflammation was shown to occur in an allogeneic T cell-dependent manner, but only became evident at 9 wk after transplant. In contrast, clinical grade acute GVHD occurs more slowly than IPS, and the delay in onset of IPS relative to GVHD suggested that the pathogenesis of IPS may be distinct from that of GVHD. However, an alternative explanation may be related to the immunosuppressive properties of macrophages in normal lung. We hypothesized that the delay in onset of IPS may be due to the kinetics of macrophage turnover in the lungs of BMT mice. AM and their precursors are relatively radioresistant (26–28), and thus, turnover of resident host macrophages in the lung occurs more slowly than in lymphocyte populations after BMT. The slow turnover of AM in the host (recipient) and the delayed kinetics for IPS in mice that were not conditioned by pretransplant irradiation (25). The delay in onset of IPS relative to GVHD suggested that the pathogenesis of IPS may be distinct from that of GVHD. However, an alternative explanation may be related to the immunosuppressive properties of macrophages in normal lung. We hypothesized that the delay in onset of IPS may be due to the kinetics of macrophage turnover in the lungs of BMT mice. AM and their precursors are relatively radioresistant (26–28), and thus, turnover of resident host macrophages in the lung occurs more slowly than in lymphocyte populations after BMT. The slow turnover of AM in the host (recipient) and the delayed kinetics for IPS could suggest that resident host AM may be inhibiting migration/activation of inflammatory cells in the lung, and that as host AM are replaced by newly differentiated donor AM, this immunosuppressive feature may be lost. This study tested the hypothesis that the delay in the kinetics of IPS may be associated with NO-mediated immunosuppression by host AM in the lungs. The results indicated that the presence of NO during the development of IPS was protective, but that this protective effect did not correlate with the turnover of lung macrophages to the donor phenotype. Rather, experiments demonstrated that the level of NO production directly correlated with the development of IPS and GVHD. Moreover, inhibition of NO production after the onset of IPS/GVHD led to rapid mortality that was dependent on the presence of T cells in the diseased mice.

Materials and Methods

Mice

Female C57BL/6 (parental donor strain, H-2b) and B6D2F1 (F1, recipient strain, H-2b,c) mice were purchased from the National Cancer Institute (Frederick, MD) and maintained in sterile microisolator cages (Lab Products, Maywood, NJ) in a sterile rodent feed and acidified water. Daily maintenance of mice was performed by the Division of Laboratory Animal Resources at the University of Kentucky, according to guidelines provided in the Animal Welfare Act. Sentinel mice were periodically screened for evidence of infection for a number of common mouse pathogens. Both the donor and recipient mice were similar in age (5–6 wk) at the onset of each study.

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

BMT models (chronic)

The chronic model of IPS and GVHD used a protocol previously described by us (22). Bone marrow cells were flushed aseptically from the femur and iliac crest of female C57BL/6 (H-2b) into RPMI 1640 (Sigma-Aldrich) medium, supplemented with 2 mM L-glutamine, 100 U penicillin, 0.1 mg/ml streptomycin (Invitrogen Life Technologies, Grand Island, NY), and 50 μM 2-ME (serum-free RPMI 1640). Erythrocytes were lysed with Tris-buffered NH4Cl (0.2M NH4Cl in Trizma base buffer, pH 7.2), and remaining BM cells were depleted of T cells by treatment with anti-Thy-1.2 (Cedarlane Laboratories, Westbury, NY) for 45 min on ice, followed by treatment with low-Tox-M rabbit complement (Cedarlane Laboratories, Westbury, NY) for 60 min at 37°C. Splenic cells used as a source of alloreactive T cells were isolated from normal C57BL/6 mice (donor) following erythrocye lysis with Tris-NH4Cl. Spleen cells were mixed with T cell-depleted bone marrow cells to give a final concentration of 5 × 10^6/ml spleen cells and 1 × 10^6/ml BM cells.Recipient B6D2F1 mice were given lethal total body irradiation (900 cGy) in a Mark 1 Cs137 irradiator (JL Shepard and Associates, Glendale, CA), and then injected i.v. with 5 × 10^6 spleen cells and 1 × 10^6/ml BM cells and 0.1 ml of PBS within 4 h of radiation conditioning. Experimental mice that developed GVHD and IPS were referred to as allo-BMT mice. Control mice that received BM cells without spleen cells and did not develop GVHD or IPS are referred to as control BMT mice. After transplantation, all mice were given trimethoprim/sulfamethoxazole in drinking water for 2 wk and housed in sterile conditions in the animal care facility at the University of Kentucky. At least four mice were sacrificed at each time point (3, 6, 9, 12 wk) for each experiment. Spleen weights were recorded, target tissues were fixed for histological analyses, and cells were stained or cultured ex vivo, as described below.

BMT model (acute)

Bone marrow isolation procedures were the same as in the chronic model, except that the number of transplanted allogeneic spleen cells was increased to 30 × 10^6 cells/mouse. In this acute model, GVHD/IPs developed more rapidly (~14 days), as evidenced by the histopathology of the target tissues (lung, liver, gut) and loss of body weight. Engraftment of donor cells in spleens of recipient mice was evaluated by flow cytometry using PE-labeled anti-H-2D and allophycocyanin-labeled anti-CD45 mAbs (Pharmingen, San Diego, CA) to determine the percentage of H-2D*-negative CD45-positive donor cells. Because the rate at which allo-BMT mice developed GVHD/IPs varied among some individuals and the severity of GVHD/IPs was shown to affect the response to NO inhibition, mice that had lost 10–15% of original body weight were selected for all NO inhibition studies. To assess the effect of NO inhibition in vivo, recipient mice were injected i.p. with aminoguanidine (AG) (800 mg/kg body weight) daily beginning at days ~2, +1, +4, +7, and +14 relative to BMT, as previously described (29). In other studies, mice were injected i.p. with the iNOS-selective inhibitor, Nω-(1-iminoethyl)-L-lysine.dihydrochloride (L-NAME) (75 mg/kg), or the nonselective NO synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester hydrochloride (75 mg/kg), on day 14 after BMT. To confirm the role of NO, allo-BMT mice were injected i.p. with the long-acting NO donor, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)aminodiazen-1-ium-1,2-diolate (1 mg/mouse), 1 h before injection with AG. Clinical symptoms were quantified for individual mice using a modification of the GVHD scoring system described by Ferrara and colleagues (30). Individual mice were scored for posture and activity, and data are presented as an average clinical score ± SEM for each treated group.
**Immunosuppression assay**

Bronchoalveolar lavage was performed with PBS containing 0.02% EDTA to isolate AM from B6D2F1 mice. Peritoneal macrophages (PM) used as control were isolated by peritoneal lavage with 5 ml of PBS-EDTA. Splenic cells (2 × 10⁶ cells/well) derived from C57BL/6 were stimulated with 0.6 μg/ml Con A in a 96-well plate. AM or PM (5 × 10⁵ cells/well) were then added into the appropriate wells. Macrophage suppressive activity in vitro was abrogated by the addition of 10 nM AG to the cell cocultures. Following incubation for 72 h at 37°C in 5% CO₂, cultures were pulsed with [³H]thymidine for the last 4 h of culture (Valent Pharmaeuticals, Costa Mesa, CA) and analyzed for proliferation in a Top-Count microplate scintillation and luminescence counter (Packard Instrument, Meriden, CT).

**NO determination**

NO production in vitro was estimated as accumulated nitrite in the culture medium after 72 h of culture in vitro at 37°C. Nitrite levels were measured spectrophotometrically at 450 nm using the Greiss reagent with sodium nitrite as standard. Absorbance was measured on a Vmax microplate reader (Molecular Devices, Sunnyvale, CA). NO production in serum was estimated using a modified Greiss assay (Cayman Chemical, Ann Arbor, MI). Serum samples were passed through a size exclusion column (Ultrafree-MC 30,000 MW exclusion; Millipore, Bedford, MA) to remove free hemoglobin. Nitrate conversion to nitrite was performed with nitrate reductase, according to recommended procedures of the manufacturer, and compared with a nitrite standard curve.

**Alanine aminotransferase (ALT) assay**

Serum was analyzed for ALT activity to determine liver injury following AG treatment, using an ALT test kit as recommended by the manufacturer. Absorbance was read at 490–520 nm, and ALT activity in Sigma-Frankel U/ml was determined from a calibration curve.

**ELISA for TNF-α**

TNF-α levels were quantified using a TNF-α ELISA kit (Pharmingen) using procedures recommended by the manufacturer. Absorbance was determined at 450 nm (μ correction 570 nm) using a Vmax microplate reader.

**Superoxide inhibition in vivo**

Extracellular superoxide was dismutated by i.p. injection of mice with 75 μU of polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) 2 h before AG administration. Intracellular superoxide was dismutated by i.p. injection of mice with 275 mg/kg membrane-permeable 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxyl (TEMPOL) 2 h before AG administration.

**ELISA for TNF-α**

TNF-α levels were quantified using a TNF-α ELISA kit (Pharmingen). ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of purified mAb (purified anti-TNFα) in coating buffer (0.2M sodium phosphate, pH 6.5, NaH₂PO₄, Na₂HPO₄). Plates were then washed three times with wash buffer (Tween in PBS) using Ultrawash Plus automatic washer/aspirator (Dynatech Laboratories, Chantilly, VA), and subsequently blocked with 0.1% BSA in PBS, pH 7.0 for 1 h at room temperature. After incubation, the plates were washed three times with wash buffer (PBS + 0.05% Tween 20). Cell-free serum samples or TNF standards were dispensed into the respective wells and incubated at room temperature for 2 h. Plates were aspirated and washed five times with wash buffer, and 100 μl of detection Ab (biotinylated anti-TNF + avidin-HRP conjugate) diluted in assay diluent buffer was added to each well. Following a 1-h incubation at room temperature, the plates were washed seven times, and 100 μl of substrate solution (tetramethylbenzidine + hydrogen peroxide) was then added to the wells. Plates were incubated at room temperature in dark for 30 min, after which stop solution (0.18 M H₂SO₄) was added to each well. Absorbance was determined at 450 nm (correction 570 nm) using a Vmax microplate reader (Molecular Devices).

**Flow cytometric analysis**

Whole lung tissue was dissected into small fragments and incubated for 45 min at 37°C in RPMI 1640 medium containing 20 U/ml collagenase and 1 μg/ml DNase I. Heat-inactivated PBS was then added to the lung cells to inactivate the activity of collagenase DNase. Single cells were released from the lung tissue by agitation in a Seward Stomacher 80 lab blender (Brinkmann Instruments, Westbury, NY). Cells were subsequently filtered through a 70-μm cell strainer (BD Discovery Labware, Franklin Lakes, NJ) and centrifuged for 10 min at 2000 rpm in a Beckman AccuSpin FR (Beckman Instruments, Palo Alto, CA) table top centrifuge to pellet the cells. Liver and spleen cells were isolated without enzymatic digestion by agitation in a lab blender. Single cells from spleen and liver were similarly filtered with cell striainers to remove debris and resuspended in complete RPMI 1640, and viable cells were counted on a hemocytometer using trypan blue.

Cells were subsequently stained with fluorochrome-labeled Abs for flow cytometric analysis. Lung, spleen, and liver cells from individual mice were analyzed using two- or three-color flow cytometry depending on each experiment. Anti-CD3 allophycocyanin, anti-CD8 PE, anti-CD4 FITC, anti-H-2Kb FITC, anti-H-2Dd PE, and rat anti-mouse CD45 allophycocyanin were purchased from Pharmingen. Rat anti-mouse F4/80 RPE Cy5 was purchased from Serotec (Raleigh, NC). Cells were resuspended at 4°C in 50 μl of PBS/1% BSA/0.01% azide solution, and 2–3 μl of the appropriate Abs was added and incubated on ice for 45 min, followed by washing once in PBS/BSA/azide solution, followed by a second wash in PBS/azide solution. The stained cells were then fixed in 1% paraformaldehyde in PBS for analysis. In some experiments, anti-FcRy (Fc block; Pharmingen) was added 15 min before the addition of labeled Abs.

**Histological analyses**

Mice were euthanized by CO₂ asphyxiation, and tissue samples from lung, liver, and colon were obtained for histological examination. Formalin-fixed tissue samples were embedded in paraffin and then sectioned and stained with H&E by the Histology Services at University of Kentucky Medical Center. Gross and microscopic examination of the stained tissues was performed to determine the changes in target organs following AG treatment.

**Determination of microvascular leakage with Evans blue dye**

Mice were injected i.v. with Evans blue dye 30 min before i.p. injection with AG. Two hours posttreatment, the lungs were flushed with PBS, pH 7, excised, rinsed in PBS, blotted, and weighed. Evans blue dye was extracted from tissues in formamide at 37°C overnight, according to the method of Mustafa et al. (31). The extract was centrifuged (7000 × g, 25 min), and the supernatant absorbance was determined at 620 (A₆₂₀) and 720 nm (A₇₂₀) on a spectrophotometer. The tissue Evans blue dye (EB) content (μg EB/g lung) was calculated by correcting A₆₂₀ for the presence of hemiglomerulus, as follows: A₆₂₀ (corrected) = A₆₂₀ - (1.426 × A₇₂₀ + 0.030), and comparing this value with a standard curve of EB in formamide.

**Blood pressure measurement**

To determine blood pressure, mice were anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg), and a catheter was inserted into femoral artery for continuous recording on a blood pressure analyzer. Mice were injected i.p. with AG (800 mg/kg body weight) once the blood pressure readings stabilized, and were then continuously monitored for an additional 2 h.

**In vivo depletion of T cells**

All Abs were used to deplete in vivo were centrifuged immediately before use at 100,000 × g for 30 min in an L-7-55 ultracentrifuge (Beckman Instruments). Anti-CD4 (GK1.5) and anti-CD8 (3.155) mAbs were prepared as ascites. Control rat ascites (SFR8) was used as a negative control. Mice were injected i.v. with 0.2 ml of either anti-CD4 or anti-CD8, 0.4 ml of anti-CD4 plus anti-CD8, or 0.4 ml of control ascites. Ab injections were performed 24 h before the administration of AG.

**Statistical methods**

Mortality curves were analyzed by log rank test, and the fraction of surviving animals at the end of a study period was analyzed by the t test. Mean time to death after initiating AG treatment on different days was analyzed by one-way ANOVA, and pairwise multiple comparison was determined by the Student-Newman-Keuls post hoc method. All other data were analyzed by an unpaired t test.

**Results**

**Evaluation of AM immunosuppressive activity during IPS development**

If the delay in onset of IPS in our B6→B6D2F1 allo-BMT model was due to replacement of NO-producing, immunosuppressive...
host AM with less suppressive bone marrow-derived donor macrophages, then it should be possible to demonstrate a loss in NO-dependent immunosuppressive activity of AM from allo-BMT mice that correlated with the development of IPS. To demonstrate an NO-dependent suppressive activity by AM, lethally irradiated B6D2F1 mice were transplanted with B6 bone marrow cells plus 5 × 10⁶ B6 spleen cells as a source of alloreactive T cells. This protocol leads to peak development of GVHD in liver, spleen, and large intestines at 3 wk post-BMT and the development of IPS by 10 wk (22). At 9 wk post-BMT, AM and PM were isolated and cocultured for 72 h with Con A-stimulated spleen cells from normal B6 mice (Fig. 1). The addition of AM from allo-BMT mice significantly inhibited the proliferation of Con A-activated spleen cells, compared with Con A-activated spleen cells alone (Fig. 1A). In contrast, the addition of PM enhanced the proliferative response. To determine whether the immunosuppressive activity of

![Graph A: Proliferation vs. NO-dependent activity](image1)

**FIGURE 1.** Immunosuppressive activity by AM and PM during IPS. Nine weeks after transplantation with C57BL/6 bone marrow cells (1 × 10⁶ cells/mouse) plus a low dose of spleen cells (5 × 10⁶ cells/mouse), AM or PM lavage cells from four individual B6D2F1 recipient allo-BMT mice were added (5 × 10⁴ cells/well) to normal C57BL/6 spleen cells (2 × 10⁵ cells/well) containing Con A (0.6 μg/ml). AG was added to the indicated wells at a final concentration of 10 mM, and cultures were incubated for 72 h at 37°C. Controls consisted of similar cultures in the absence of macrophages. A, Cultures were pulsed with [³H]thymidine during the last 4 h of culture to measure spleen cell proliferation. Dotted line represents the proliferation observed for normal spleen cells alone cultured in the presence of AG. B, Culture supernatants were collected after 72 h, and nitrite levels were determined (n.d., not detected). Data presented are representative of at least three separate experiments. *, p < 0.001.
AM from allo-BMT mice was due to the production of NO. AG, a specific inhibitor of iNOS, was added to identical cultures of Con A-stimulated spleen cells plus AM or PM. The addition of AG to cocultures of Con A-activated spleen cells plus AM completely reversed the inhibition of spleen cell proliferation, indicating that NO was the immunosuppressive factor released from AM. Culture supernatants from cocultures of AM plus spleen cells also contained elevated levels of nitrite, indicating NO production by the AM from allo-BMT mice; however, the addition of AG completely inhibited NO production by AM (Fig. 1B). AG also increased proliferation of cultures containing PM even though nitrite levels were barely detectable in culture supernatants, indicating that even low levels of NO production by PM can prevent full activation of spleen cells by Con A.

To determine whether the immunosuppressive activity of AM diminished during the course of IPS development, AM were collected from allo-BMT mice at 3, 6, 9, and 12 wk after transplant and evaluated for the ability to inhibit Con A-induced spleen cell proliferation (Fig. 2). AM from allo-BMT mice had substantially greater inhibitory activity than AM from normal mice. In contrast, AM from control BMT mice never displayed immunosuppressive activity greater than AM from normal mice. However, the level of immunosuppression by AM from allo-BMT mice was elevated at all time points following allo-BMT, indicating that a change in the suppressive activity by AM could not account for the delayed development of IPS relative to the onset of GVHD in other tissues.

The contribution of inducible NO synthesis to the development of IPS and GVHD

To investigate the role of NO in the development of GVHD/IPS, a 6-day acute GVHD model was established in which AG could be administered daily to transplanted mice during the development of disease. As an index for NO production, sera were evaluated for the levels of nitrite/nitrate following reduction of nitrate by nitrate reductase. Daily treatment of allo-BMT mice with AG beginning 1 day after BMT significantly reduced serum levels of nitrite/nitrate (Fig. 3) and reduced the signs of GVHD and IPS, including splenomegaly, serum TNF-α, and lymphocyte inflammation in the lung (Table I). Histological analysis of lung and liver indicated reduced inflammatory cell infiltration in AG-treated allo-BMT mice compared with untreated allo-BMT mice (data not shown). Control BMT mice did not display elevated serum nitrite/nitrate levels and never developed signs or symptoms of GVHD/IPS. Despite the apparent protective effects of AG treatment on GVHD/IPS in allo-BMT mice, AG-treated allo-BMT mice lost significantly more body weight than untreated allo-BMT mice (data not shown) and experienced a significant increase in mortality compared with vehicle control-treated allo-BMT mice (Table I). AG-enhanced mortality in allo-BMT mice was not due to general toxicity, because AG-treated BMT control mice did not display any enhanced morbidity or mortality. These observations suggested that in addition to detrimental effects of NO production, NO also served a protective role that reduced an unknown lethal effect of GVHD/IPS.
Characterization of NO-enhanced mortality in mice with IPS and GVHD

Because mortality appeared to be linked in some way with the development of GVHD, we next determined whether the severity of GVHD had an impact on the AG-enhanced mortality (Fig. 4A). Allo-BMT mice that did not receive any additional treatment survived through day 50 posttransplant. However, if daily administration of AG was begun 1 day after transplant, mice only survived through day 21. If GVHD was allowed to develop for 7 days before initiation of daily AG administration, mice only survived an additional 2 days (day 9 posttransplant). Finally, if AG treatment was initiated 14 days after allo-BMT, all mice displayed signs of morbidity by 2 h after AG administration and none survived beyond 24 h after AG injection. Daily administration of AG to mice was not generally toxic, because BMT control mice that received daily injections with AG showed no morbidity or mortality throughout the entire study period. Calculation of the mean time to death indicated that survival following initiation of AG treatment was significantly reduced when administration of AG was delayed until the onset of GVHD/IPS (Fig. 4B). The results demonstrate that mortality following AG treatment directly correlated with disease progression and severity of GVHD/IPS.

Several possible mechanisms by which AG treatment may have enhanced mortality were evaluated, including inhibition of bone marrow engraftment, enhanced liver injury, or an AG-induced increase in blood pressure (Table II). Daily injections with AG beginning 1 day after transplant caused a significant reduction in engraftment of donor hemopoietic cells in control BMT mice 14 days after transplant compared with control BMT mice without AG treatment. In contrast, allo-BMT mice did not show any significant reduction in the percentage of donor cells at day 14, compared with allo-BMT mice without AG treatment. This indicated that a failure of bone marrow to engraft could not be the cause of AG-enhanced mortality in allo-BMT mice. Injection of allo-BMT or control BMT mice with AG 14 days after transplant did not elevate serum level of ALT 2.5 h after injection, when morbidity was evident, suggesting that AG-enhanced mortality was not due to massive liver injury (Table II). NO is also known to regulate blood pressure via its vasodilatory activity. To determine whether inhibition of NO by AG treatment caused a hypertensive response that could contribute to mortality, blood pressure was continuously monitored in mice for 2 h following AG injection (Table II). Untreated normal mice maintained a constant blood pressure of 78–80 mmHg over the observation period. As expected, day 14

Table 1. AG treatment beginning on day 1 decreases signs of GVBD/IPS, but increases mortality

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control BMT</th>
<th>Control BMT + AG</th>
<th>Allo-BMT</th>
<th>Allo-BMT + AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight (mg) (day 14)</td>
<td>82 ± 11</td>
<td>58 ± 32</td>
<td>48 ± 7</td>
<td>103 ± 22</td>
<td>45 ± 19a</td>
</tr>
<tr>
<td>Serum TNF-α (pg/ml) (day 14)</td>
<td>ND</td>
<td>0.0 ± 0.0</td>
<td>13.6 ± 8.4</td>
<td>136.2 ± 38.5</td>
<td>50.3 ± 9.5a</td>
</tr>
<tr>
<td>Lung lymphocytes (day 14) (×10⁶) CD4⁺</td>
<td>2.4 ± 1.6</td>
<td>1.4 ± 0.1</td>
<td>0.8 ± 0.4</td>
<td>19.6 ± 5.6</td>
<td>9.2 ± 0.8a</td>
</tr>
<tr>
<td></td>
<td>2.0 ± 1.8</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>33.8 ± 11.9</td>
<td>22.5 ± 1.5a</td>
</tr>
<tr>
<td>Mortality (day 28)</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>2/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

a Value of p < 0.05 when compared with allo-BMT without AG treatment.

b Value of p < 0.01 when compared with allo-BMT without AG treatment.

c Not significant.
control BMT mice experienced a rise in blood pressure from 73 to 90 mmHg over the same time frame. In contrast, day 14 allo-BMT mice had a reduced pressure (44 mmHg) before AG treatment, which progressively fell to 28 mmHg before mortality occurred. Thus, inhibition of the vasodilatory activity of NO could not be the cause of AG-enhanced mortality.

To determine whether AG treatment was altering the levels of NO in vivo in allo-BMT mice, serum nitrite/nitrate levels were assayed 2.5 h after administration of AG in allo-BMT and control BMT mice on day 14 posttransplant (Fig. 5). As seen before, serum nitrite/nitrate levels were significantly higher in allo-BMT mice compared with control BMT. AG administration significantly reduced serum nitrite in allo-BMT mice 2.5 h after administration to the level seen in control BMT, indicating that the AG-enhanced mortality correlated with a reduction in NO production in vivo. To evaluate whether AG-enhanced mortality was caused by the inhibition of NO

Table II. Effect of AG treatment beginning on day 1 on engraftment, liver injury, and blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control BMT</th>
<th>Control BMT + AG</th>
<th>Allo-BMT</th>
<th>Allo-BMT + AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of engraftment (day 14)</td>
<td>–</td>
<td>31.1 ± 9.0</td>
<td>19.6 ± 6.6</td>
<td>72.4 ± 9.3</td>
<td>67.8 ± 6.8*</td>
</tr>
<tr>
<td>Liver injury (day 14) (serum ALT, U/ml)</td>
<td>47.2 ± 18.6</td>
<td>18.4 ± 21.9</td>
<td>38.8 ± 11.8</td>
<td>52.6 ± 8.1</td>
<td>33.4 ± 3.9*</td>
</tr>
<tr>
<td>Arterial blood pressure change (mmHg) (day 14)</td>
<td>78 → 80</td>
<td>73 → 90</td>
<td>44 → 28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Not significant.

Value of $p < 0.01$ when compared with allo-BMT without AG treatment.
levels rather than nonspecific effects of AG, allo-BMT mice were injected with the long-acting NO donor, NOC-18, 1 h before AG administration. NOC-18 pretreatment significantly reduced the morbidity and mortality caused by AG treatment in allo-BMT mice (Fig. 6), indicating that the inhibitory effect of AG on NO synthesis was responsible for enhanced mortality in allo-BMT mice.

Yang et al. (8) have also observed an increase in mortality in NOS2−/− mice following allogeneic BMT. These investigators suggested that the increased mortality may have been due to an elevated level of superoxide, because in the absence of NO, superoxide molecules would not be diverted into the generation of the short-lived peroxynitrite molecule. To test whether extracellular superoxide levels contributed to the enhanced mortality following AG treatment of allo-BMT mice, day 14 transplanted mice were administered PEG-SOD 2 h before the administration of AG. Lung tissues were analyzed for lipid peroxidation 2.5 h after AG treatment, as an indication of superoxide generation (Fig. 7A). Treatment of day 14 allo-BMT mice with AG significantly increased the level of lipid peroxidation in lung, which was in agreement with the studies of Yang et al. (8). Administration of PEG-SOD before AG injection prevented the increase in lipid peroxidation following inhibition of NO, indicating that PEG-SOD dismutated the superoxide anion that was generated following AG treatment. However, pretreatment of allo-BMT mice with PEG-SOD failed to protect mice from the AG-enhanced mortality (Fig. 7B), indicating that increased extracellular superoxide levels following AG administration were not responsible for enhanced mortality. Because it was possible that intracellularly generated superoxide may have contributed to AG-enhanced mortality, a similar study was performed in which allo-BMT mice were pretreated with the cell-permeable SOD mimetic, 4-hydroxy-TEMPO (275 mg/kg) 1 h before administration of AG. Pretreatment with 4-hydroxy-TEMPO failed to rescue allo-BMT mice from mortality following AG administration, confirming that elevated superoxide levels were not the cause of AG-enhanced mortality (data not shown).

To determine whether AG-enhanced mortality could be associated with cytokine-mediated vascular shock syndrome, serum TNF-α levels were also measured 2.5 h after administration of AG in day 14 allo-BMT and control BMT mice (Fig. 8). Serum TNF-α levels were significantly higher in allo-BMT mice compared with control BMT mice. In contrast to serum nitrite/nitrate levels, the elevated level of serum TNF-α in allo-BMT mice was not reduced following AG treatment, but rather showed a moderate, nonsignificant increase compared with untreated allo-BMT mice. To confirm that a vascular leak syndrome was not the cause of AG-enhanced mortality, lung permeability (as measured by the Evans blue technique) was measured 2.5 h after AG in allo-BMT and control BMT on day 14 posttransplant. Vascular permeability in the lungs was not significantly increased in allo-BMT mice compared with control BMT either before or after AG administration (data not shown). These data indicated that development of a vascular shock syndrome was not a likely cause of AG-enhanced mortality.

Contribution of T cells to AG-enhanced mortality in mice with GVHD/IPS

Because AG-enhanced mortality was only observed in transplanted mice that received allogeneic spleen cells as a source of mature alloreactive T cells, individual subsets of T cells were depleted with specific Ab in day 14 allo-BMT mice to determine whether the presence of T cells at the time of AG administration could contribute to the enhanced mortality (Fig. 9). Fourteen days after transplant, allo-BMT mice were injected with anti-CD4, anti-CD8, anti-CD4 plus anti-CD8, or rat Ig control Ab. Twenty-four hours later, mice were injected with AG. Over the subsequent 24 h,
untreated allo-BMT mice displayed no mortality, whereas only 3 of 13 AG-treated allo-BMT mice survived. In contrast, 13 or 15 allo-BMT mice that were previously injected with anti-CD4 survived following AG treatment, which was significantly different from both AG-treated allo-BMT and AG-treated allo-BMT that were injected with control Ig. Combined injection with anti-CD4 plus anti-CD8 partially protected mice from AG-enhanced mortality, which was significantly different from AG-treated allo-BMT that were injected with control Ig and approached significance when compared with AG-treated allo-BMT mice. Prior injection with anti-CD8 Ab did not significantly protect allo-BMT mice from AG-enhanced mortality. These studies indicated that NO synthesis during ongoing GVHD/IPS provided a protective effect that appeared to be directed against CD4+ T cells, which, if removed, permitted substantially greater morbidity and mortality during GVHD/IPS.

Discussion

The primary immunosuppressive molecule produced by activated macrophages is NO. NO is a free radical derived from the oxidation of L-arginine by a process that is catalyzed by iNOS and two constitutive enzymes, endothelial and neuronal NOS (eNOS and nNOS, respectively). The effects of NO are pleiotropic and include regulation of vascular homeostasis and neurotransmission. Furthermore, NO is involved in the effector functions of macrophages and endothelial cells. Excessive NO production by activated macrophages is thought to cause damage to target organs during GVHD development (32–34). In addition, NO can act as a physiological scavenger of superoxide anions (35). NO reacts with superoxide anions to generate peroxynitrite, a short-lived potent oxidizing molecule known to cause tissue damage (36–38). In this study, we evaluated the role of NO during development of GVHD and IPS. The approach we took was to determine the effect of AG, a selective iNOS inhibitor, on the survival of mice developing GVHD. This was based on the premise that we could prevent excessive NO production caused by iNOS during disease development without impairing the normal physiological processes regulated by constitutive NOS. Our results demonstrated that inhibition of NOS activity with AG resulted in significant mortality in allo-BMT mice, but not in the control BMT group. These results suggested that NO serves a protective role during GVHD/IPS development, and that increased NO production may be associated with limiting disease progression. To rule out any nonspecific effects of AG that may be unrelated to inhibition of NO generation, studies were performed in which AG-treated allo-BMT mice were pretreated with a long-acting NO donor, NOC-18. Pretreatment with NOC-18 significantly reversed the effects of AG in allo-BMT mice, confirming that NO generation during GVHD/IPS was serving a protective function. Although AG is reported to be a selective iNOS inhibitor, its selectivity is not absolute, and at higher doses can also inhibit eNOS and nNOS (39). Thus, we cannot exclude that the protective effects of NO that may be unrelated to inhibition of NO generation, studies were performed in which AG-treated allo-BMT mice were pretreated with a long-acting NO donor, NOC-18. Pretreatment with NOC-18 significantly reversed the effects of AG in allo-BMT mice, confirming that NO generation during GVHD/IPS was serving a protective function. Although AG is reported to be a selective iNOS inhibitor, its selectivity is not absolute, and at higher doses can also inhibit eNOS and nNOS (39). Thus, we cannot exclude that the protective effects of NO are derived only from iNOS. In fact, studies on the protective effects of NO in intestinal inflammation have clearly indicated a role for eNOS in regulating inflammatory cell-mediated tissue injury (40). Preliminary studies using more selective inhibitors of iNOS, L-NIL and 1400W, have yielded equivocal results in that L-NIL also caused increased morbidity/mortality in allo-BMT mice, but the most selective iNOS
inhibitor, 1400W, was without significant effect. Although these initial observations suggest that eNOS and/or nNOS may contribute to the generation of protective NO, additional studies are needed to confirm their roles.

Because increases in circulating TNF-α in vivo are often associated with vascular leakage and increased mortality, it was important to determine whether the protective effect of NO in allo-BMT was regulating the release of TNF-α. However, TNF-α levels were not significantly elevated in allo-BMT mice following AG treatment, and analysis of pulmonary vascular permeability failed to identify any increase in vascular leakage following AG administration to allo-BMT mice that had developed GVHD/IPS. A recent study by Yang et al. (8) demonstrated the allogeneic BMT in iNOS knockout mice preconditioned with total body irradiation cyclophosphamide had elevated mortality, but comparable lung inflammation compared with transplanted wild-type mice. It was proposed that the lack of NO generation allowed for increased oxidant injury, because NO was not available as a scavenger of superoxide. We observed a significant decrease in the numbers of inflammatory cells in lung, liver, and colon if AG administration was begun 1 day following transplant; however,

**FIGURE 7.** Contribution of reactive oxygen to AG-enhanced mortality. Groups of six allo-BMT mice received a single i.p. injection of AG (800 mg/kg) or were left untreated on day 14 after transplantation with C57BL/6 bone marrow cells (1 x 10⁷ cells/mouse) plus a high dose of spleen cells (30 x 10⁶ cells/mouse). One group was injected with PEG-SOD 2 h before AG administration. A, Livers from individual mice were collected 2.5 h after AG injection, and tissue extracts were evaluated for lipid hydroperoxide content. Data are representative of three individual experiments. B, In a separate study, groups of mice as in A were evaluated for percentage of mortality at 24 h after AG administration. Values represent number of deaths/number of mice in a group. Data are representative of two individual experiments. *, p ≤ 0.05 compared with allo-BMT group. n.s., Not significant compared with allo-BMT group.
these mice also experienced higher mortality compared with allo-BMT in the absence of AG treatment. The reason for the difference in GVHD/IPS inflammation is not readily apparent; however, differences in preconditioning, strain combination (B10.BR→C57BL/6), or method of inducing NO deficiency could contribute to the observed difference in outcomes.

Studies by us and others (25, 41, 42) have shown that the development of IPS in the setting of GVHD is dependent on the presence of mature, alloreactive T cells in the donor marrow. Moreover, studies in this report demonstrated that the kinetics of mortality following AG administration correlated with the severity of GVHD/IPS at the time of AG treatment initiation. Based on these observations, we performed T cell depletion in vivo before AG administration in allo-BMT mice with ongoing GVHD/IPS. Surprisingly, prior depletion of CD4\(^+\) T cells significantly protected allo-BMT mice from the effects of AG administration. CD8\(^+\) T cell depletion also partially protected allo-BMT mice from AG-enhanced mortality. Thus, it appeared that the production of NO in vivo during the development of GVHD/IPS offered allo-BMT mice protection against some form of T cell-dependent injury. The nature of this T cell-mediated injury that is inhibited by NO production in vivo is not known at the present time; however, several possibilities exist.

NO is a potent inhibitor of T cell proliferation, but the speed at which day 14 allo-BMT mice died following AG administration suggests that mortality dependent on an increased number of T cells is an unlikely mechanism. Although NO can inhibit T cell proliferation and can modulate development of Th1 and Th2 immunity, the ability of NO to inhibit effector functions of mature T cells is less clear. In the context of results in this study, NO synthesis during GVHD/IPS would need to regulate secretion of cytokines and/or inhibit cytotoxic activity of mature T cells. However, the bulk of evidence in the current literature does not support a role for NO in the inhibition of effector function by T cells.

Interestingly, NO is known to block caspase activity by S-nitrosylation of enzymatic active sites (43). NO-mediated caspase inhibition inhibits the processing of IL-1\(\beta\) and IL-18 procytokines via IL-1\(\beta\)-converting enzyme and also blocks apoptosis by inhibiting the activity of several caspase enzymes (44, 45). It may be possible that inhibition of NO synthesis by AG allows for rapid processing of preformed cytokines in allo-BMT mice with GVHD/IPS, leading to increased IL-1 and IL-18, which directly or indirectly affects mortality. However, the manner in which T cells would be involved in such a mechanism is uncertain. The cytotoxic capacity of alloreactive T cells or NK cells could be affected by high in vivo levels of NO that occur during GVHD, because granzyme-dependent cytolyis has been shown to use caspase activation pathways (46). Alternatively, the target cells for CTLs may be more resistant to cytosis in the presence of high concentrations of NO, because several studies have shown that NO can mediate antipapoptotic effects via its inhibitory activity on caspases involved in apoptotic pathways (45, 47, 48). Preliminary studies in our lab have suggested that T cell-dependent toxicity can be adoptively transferred from allo-BMT mice into BMT control mice (data not shown). These preliminary studies have shown that injection of day 14 BMT control mice with T cells from day 14 allo-BMT mice did not cause morbidity in recipient mice unless AG is also administered. Additional studies with this new adoptive transfer model will enable us to evaluate mechanisms by which T cells from mice with ongoing GVHD can contribute to rapid mortality in the absence of NO and to identify how NO provides a protective effect against alloreactive T cells during this inflammatory disease.

Several nonimmunologic effects could also contribute to the enhanced morbidity/mortality seen after AG administration. For example, a reduction of vasodilatory effects of NO in vivo would be expected to promote vasoconstriction and elevated blood pressure. Evidence for this hypertensive effect was seen in BMT control mice following AG injection. However, continuous monitoring of blood pressure in allo-BMT mice following AG administration demonstrated a progressive drop in blood pressure in treated mice, indicating that vasoconstrictive effects were not a likely cause of the increased mortality. NO is also known to have potent anti-thrombotic activity via its ability to inhibit platelet activation, adhesion, and aggregation (49, 50). Much of the effect of NO on platelets is derived from NO released by endothelial cells via the eNOS gene. Whether the dose of AG used in our studies has some nonselective inhibitory effects on NO synthesis by endothelial cells or whether iNOS-derived NO is serving an important anti-thrombotic role in allo-BMT mice is uncertain. Although AG is a selective inhibitor of NOS2, the relatively high dose used in our studies.
may have increased the possibility of inhibition of eNOS activity, as has been previously observed (39, 51). It should be noted that administration of AG at a lower dose of 200 mg/kg also caused high mortality in day 14 allo-BMT mice (data not shown); however, possible nonselective effects by AG need further study. Preliminary analysis of histological sections of tissues from day 14 allo-BMT mice 2.5 h after AG administration did not show evidence of massive vascular thrombosis; however, additional studies will be needed to fully evaluate this possibility, because microthrombi could also affect viability in the mice. Although AG shows selective inhibition of iNOS compared with eNOS, the inhibitory dose for nNOS is nearly identical with that for iNOS (51). Finally, consideration of any effect of AG on eNOS, nNOS, or other nonimmunologic process as a possible cause of enhanced mortality in GVHD/IPS mice must also take into consideration a role of T cells given the results of T cell depletion studies in this study.

In conclusion, NO generation during the development and progression of GVHD/IPS appears to serve a protective role in mice. An enhanced level of mortality was observed in allo-BMT mice when NO generation was inhibited after the development of GVHD/IPS. The protective role of NO was directed against effector functions of T cells in mice with GVDH/IPS. Additional studies are required to fully delineate the T cell-dependent mechanisms involved and how NO generation during GVHD/IPS modulates the injurious effects by T cells.

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References


