An Experimental Model of Idiopathic Pneumonia Syndrome After Bone Marrow Transplantation: I. The Roles of Minor H Antigens and Endotoxin

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Idiopathic pneumonia syndrome (IPS) refers to diffuse, noninfectious pneumonia that occurs after allogeneic bone marrow transplantation (BMT). We have developed a model of IPS using a well-characterized murine BMT system (B10.BR → CBA) in which lung injury after BMT can be induced by minor histocompatibility (H) antigenic differences between donor and host. Lung pathology and broncho-alveolar lavage (BAL) fluid were analyzed in transplant recipients before and after both syngeneic and allogeneic BMT. At 2 weeks after BMT, no specific pathologic abnormalities were noted; at 6 weeks, both pneumonitis and mononuclear cell infiltration around vessels and bronchioles were observed only in mice receiving allogeneic BMT. This injury was associated with elevated BAL fluid levels of endotoxin (lipopolysaccharide [LPS]), neutrophils, and tumor necrosis factor α. No pathologic organisms were isolated from the respiratory tract of any animal. We also tested the role of endotoxin in the development of this injury. Injection of LPS 6 weeks after transplantation caused profound lung injury only in mice with moderate graft-versus-host disease; dramatic increases in BAL neutrophils and tumor necrosis factor α were observed, with alveolar hemorrhage occurring in 4 of 12 of these mice but in no other group. We conclude that (1) this murine BMT system is a potentially useful model of clinical IPS; (2) minor H differences between donor and recipient can be important stimuli in the pathogenesis of IPS; and (3) endotoxin in BAL fluid is associated with lung injury, and excess endotoxin can cause the development of alveolar hemorrhage in this model.

THE USE OF HLA-identical bone marrow transplantation (BMT) is an important therapeutic option for treating a number of hematologic, malignant, and genetic disorders, but it is accompanied by many serious complications, including pulmonary toxicity. The lung has long been identified as an organ susceptible to injury after clinical BMT. Pulmonary insults in various forms occur in 25% to 55% of transplanted patients and account for approximately 40% of transplantation-related mortality.[1-3] In a recent review, pneumonia was identified as the primary cause of death in 224 of 572 BMT patients over a 16-year period, with no infectious pathogen isolated in approximately 50% of the cases.[4] This form of noninfectious posttransplantation lung injury has been named idiopathic pneumonia syndrome (IPS) by a recent National Institutes of Health panel.[1] Diagnostic criteria of IPS include evidence of nonlobar radiographic infiltrates, signs and symptoms of pneumonia (eg, fever, cough, dyspnea, and rales), evidence of abnormal pulmonary physiology (hypoxemia and altered pulmonary function testing), and the absence of infectious organisms in sputum, broncho-alveolar lavage (BAL) fluid, or biopsy specimens. The most frequently reported histologic pattern that corresponds to this clinical picture is interstitial pneumonitis, historically used interchangeably with IPS. However, several other findings have been reported including vasculitis, bronchiolitis, cellular atypia, edema, alveolar exudate or hemorrhage, and hyaline membranes.[1,5-7] The median time to onset of IPS is 42 to 49 days after BMT (range, 14 to 90 days), with associated mortality rates of 50% to 60%.[1,2]

In 1973, Neiman et al[8] published the first comprehensive report on interstitial pneumonitis (IP) after BMT and noted its association with allogeneic marrow grafts (in contrast to autologous or syngeneic grafts) and with graft-versus-host disease (GVHD). In patients receiving transplants for severe aplastic anemia with an HLA-identical family donor, acute GVHD was a significant risk factor for all pneumonias and was the single greatest risk factor for IPS.[9] In 1978, Beschorner et al[10] noted an association between severity of clinical GVHD and a histologic pattern consistent with lymphocytic bronchitis found on postmortem exams. This finding was not seen in patients who received autologous BMTs or in untransplanted controls.[10] A linear correlation between the onset of acute GVHD and respiratory symptoms was also cited, an association that was verified in some reports[3,4,11] but not in others in which IP was also seen after autologous and syngeneic BMT.[12,13] In addition, the histologic pattern of lymphocytic bronchitis that was thought to represent a graft-versus-host reaction of the lung was not reproduced in subsequent reports.[5-7,14]

Although the frequent association of acute GVHD with IPS suggests the importance of alloreactivity in its development, a causal relationship between the two has not been clearly established. Currently, there is controversy as to whether the lung should be considered a target organ in acute GVHD. Epithelial cell damage, considered pathognomonic for acute GVHD in other organs, has not been consistently identified among the myriad of histologic findings noted in the lungs of patients with IPS. This heterogeneity of pulmonary histopathology is further exacerbated by suboptimal quality and quantity of specimens due to the timing of and the significant risks associated with biopsy procedures. We have developed an experimental model of IPS using a well-characterized murine BMT system across minor histocom-

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patibility (H) differences.[15-18] The number of BM and T cells injected into recipient mice was chosen to produce significant GVHD in the majority of allogeneic recipients by 6 weeks after BMT, a time that coincides with the median time of onset of clinical IPS.[1] We have used this model to explore the relationships between systemic GVHD, endotoxin, and pulmonary pathology. Our data show that minor H antigens can stimulate the development of IPS even when systemic GVHD is mild. They also point to an important role for endotoxin, the presence of which in the serum and BAL fluid is associated with pulmonary pathology. Endotoxin challenge intensifies the lung injury in animals with significant GVHD and produces alveolar hemorrhage only in this context.

MATERIALS AND METHODS

Mice and BMT. Female CBA/J (H-2^k) and B10.BR (H-2^k) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and received transplants between the ages of 10 and 14 weeks. BM cells (5×10^6) harvested from the femurs and tibias of donor CBA or B10.BR mice were supplemented with 1×10^5 nylon wool nonadherent donor splenic T cells. Cell mixtures were resuspended in Leibovitz's L-15 medium (Life Technologies, Grand Island, NY) and transplanted into CBA recipients via tail vein infusion (0.25 mL total volume). Before transplantation, host mice received 11 Gy of total body irradiation (^{137}Cs source) delivered in two fractions separated by 3 hours to reduce gastrointestinal toxicity. This dose of irradiation has been shown not to cause histologically detectable pulmonary injury in normal CBA mice.[17] Mice were subsequently housed in sterilized micro-isolator cages and received normal chow and autoclaved hyperchlorinated water for the first 2 weeks after BMT and filtered water thereafter. In some experiments, mice were injected intravenously with lipopolysaccharide (LPS), *Escherichia coli* serotype 026:B6 (Sigma, St Louis, MO).

Assessment of GVHD. The severity of GVHD was assessed by the percentage of weight change, a parameter that has been found to be a reliable indicator of systemic GVHD in this and several other murine models.[19-21] Transplanted mice were ear punched and individual weights were obtained and recorded on day +1 and weekly thereafter until the time of analysis or LPS administration (18 to 36 hours before analysis). Weight loss of greater than 10% has been considered indicative of significant GVHD in previous reports.[19-21] In this study, a threshold of 10% weight loss was used to signify the presence of moderate GVHD. In addition, the degree of systemic GVHD was assessed by a scoring system described in Table 1 that incorporates five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. At the time of analysis, mice from coded cages were evaluated and graded from 0 to 2 for each criterion. A clinical index was subsequently generated by summation of the five criteria scores (maximum index = 10).

BAL, cell surface phenotyping, and cellular differential. At the time of analysis, mice were killed by exsanguination and BAL was performed. Through an incision and dissection of the anterior neck, a 19-gauge tubing adapter (used as a tracheostomy tube) was surgically inserted into the trachea and secured with silk suture. A 0.8-mL aliquot of 1× phosphate-buffered saline (PBS) containing 0.6 mmol/L EDTA was instilled into the lungs through the secured tracheostomy tube, of which 0.7 mL was removed and placed into a sterile tube on ice. This procedure was repeated nine additional times, with subsequent aliquots combined in a second tube. When BAL fluid was evaluated for endotoxin concentration, pyrogen-free PBS (<0.005 EU/mL) was used (Bio Whittaker, Walkersville, MD). The tubes were centrifuged at 1,500 rpm for 5 minutes, and supernatant from the first tube was frozen for subsequent analysis of cytokine and endotoxin concentrations. Cell pellets from both tubes were combined, washed twice, and counted. Aliquots of 4×10^5 cells were washed two additional times with 2% fetal bovine serum (FBS) in PBS. Fc receptors were blocked with monoclonal antibody (MoAb) 2.4G2[22] at 4°C for 15 to 20 minutes. Cells were then incubated at 4°C for 30 minutes with fluorescein isothiocyanate– or R-phycoerythrin–conjugated MoAbs to cell surface markers Mac-1, CD4, and CD8 (Pharmingen [San Diego, CA] and Boehringer Mannheim [Indianapolis, IN]). Cells were then washed twice, fixed in 0.5 mL of 1% paraformaldehyde in PBS, and analyzed on a FACScan system (Becton Dickinson, San Jose, CA). Aliquots of cell suspensions (2×10^6 cells/mL) were placed on glass cover slips, air-dried, stained with Wright-Giemsa, and mounted on microscope slides. Coded slides were then evaluated visually for morphologic differentials.

Table 1. Assessment of Clinical GVHD in Transplanted Animals

Criteria	Grade 0	Grade 1	Grade 2
Weight loss	<10%	>10% to <25%	>25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin

CBA mice received allogeneic or syngeneic bone marrow and T cells as described in the Materials and Methods. Mice were ear tagged on day 0 and evaluated at the time of analysis for evidence of GVHD by assessment of five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Individual mice from coded cages received a score of 0 to 2 for each criteria (maximum score of 10), as described above.

Tissue procurement and semiquantitative histopathology. After BAL, lungs from each mouse were inflated with 1 mL of Tissue Tek OCT compound (Miles, Elkhart, IN) and removed from the thoracic cavity. The caudal halves of the right lower lobe and left lung were immersed in 10% buffered formalin. Formalin-preserved sections were embedded in paraffin, cut into 5-μm thick sections, and stained with hematoxylin and eosin (H & E) for histologic examination. Slides were coded without reference to mouse type or prior treatment status and examined by a pathologist (L.K.) to establish an index of injury. In some animals, a portion of the right lobe of the liver was also harvested in buffered formalin, paraffin-embedded, stained with H & E, and examined in a coded fashion by a pathologist (J.M.C.). Hepatic tissue pathology was scored semiquantitatively using a detailed system previously described.[16,23]

Cytokine and endotoxin determination. Concentrations of tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) were measured in BAL fluid supernatant (obtained from the first lavaged aliquot) by sandwich enzyme-linked immunosorbent assay (ELISA) using specific antimurine MoAbs for capture and detection and the appropriate standards purchased from Genzyme (Cambridge, MA). Assays were performed according to the manufacturer's protocol; in addition, samples were diluted 10% (vol/vol) with 1 mg/mL of bovine serum albumin (BSA) in PBS to a final concentration of 0.1 mg/mL of BSA per sample. Standards were diluted in 0.1 mg/mL BSA in PBS. Lower limits of detection of these assays were 10 to 20 pg/mL. For determination of endotoxin concentration in BAL fluid and serum, the limulus amebocyte lysate (LAL) assay QCL-1000 test kit was used (Bio Whittaker). Assays were performed according to

the manufacturer's protocol. Briefly, serum and BAL fluid samples were collected and analyzed using pyrogen-free materials, diluted 10% (vol/vol) in LAL reagent water, and heated to 70°C for 5 minutes to remove any nonspecific inhibition to the assay. The lower limit of detection was 20 pg/mL for serum (using a 10-minute incubation time) and 2 pg/mL for BAL (using a 30-minute incubation time, which increased the sensitivity of the assay). In all cytokine and endotoxin determinations, samples and standards were run in duplicate.

Statistical considerations. Data presented have been obtained from 83 animals receving transplants in eight separate experiments, with 29, 23, and 31 mice in syngeneic, mild GVHD, and moderate GVHD groups, respectively. Because it was not possible to test every parameter in each animal undergoing transplantation, mice were chosen randomly for analysis to allow for sample sizes of 9 to 12 per group in the majority of evaluations. All values are expressed as the mean ± SEM. Statistical comparisons between groups were made using the nonparametric unpaired Mann-Whitney Test, except where binomial outcomes were analyzed, in which cases the Fisher's Exact Test was used.

RESULTS

The development of significant lung histopathology 6 weeks after transplantation correlates with the presence but not severity of GVHD. CBA mice were transplanted with syngeneic (CBA) or allogeneic, MHC-identical (B10.BR) BM and T cells as described in the Materials and Methods. Transplant parameters were chosen so that the majority of mice would be available for analysis 6 to 7 weeks after BMT, when IPS has its peak clinical incidence,[1] and a significant percentage of animals would have clinically evident GVHD. When allogeneic BMT recipients were evaluated at 6 weeks after BMT in pilot experiments, approximately 60% of the animals showed weight loss of at least 10%, a threshold previously described for clinically important GVHD in this model.[21] In subsequent experiments, animals were individually ear punched, weighed weekly, and, 6 weeks after BMT, classified according to their percentage of weight loss. Surprisingly, as a group, allogeneic BMT recipients with less than 10% weight loss were not distinguishable in this respect from syngeneic controls at 4 and 6 weeks after transplantation (Fig 1A). To delineate GVHD with greater precision in this group, a clinical scoring system was developed that also included fur texture, skin integrity, posture, and activity (Table 1). As shown in Fig 1B, this semiquantitative assessment showed that clinical scores of allogeneic mice with less than 10% weight loss differed significantly from scores of both syngeneic BMT recipients and allogeneic mice with greater than 10% weight loss. This difference in clinical GVHD persisted independent of weight loss (data not shown, $P = .001$). Therefore, in these analyses, allogeneic BMT recipients were grouped according to the severity of GVHD (mild or moderate), based on weight loss as well as a clinical score.

Mice were killed at 2 and 6 weeks after BMT and detailed histopathologic analysis of the lungs was performed. At 2 weeks, no histologic abnormalities were noted in either the syngeneic or allogeneic group. At 6 weeks, lungs of mice receiving syngeneic transplants maintained virtually normal histology (Fig 2A), but two major abnormalities were apparent in the allogeneic group (Fig 2B through F). First, a dense

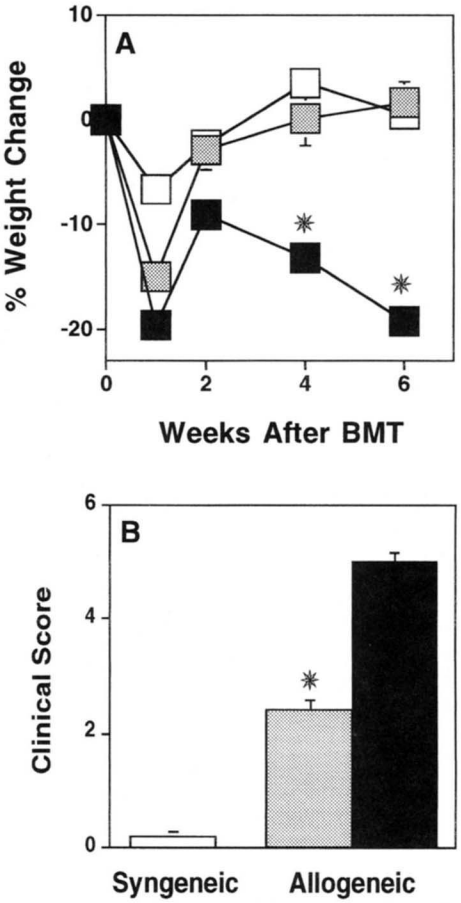

Fig 1. (A) Percentage of weight change in mice after BMT. CBA mice received allogeneic (■, ■) or syngeneic (□) BMT as described in the Materials and Methods. Transplanted mice were ear tagged and weighed on day +1 and then weekly up to and including the day of analysis. Six weeks after transplantation, recipients of allogeneic BMT were divided into two groups, those with moderate GVHD (■; >10% weight loss) and mild GVHD (■; <10% weight loss). At 4 and 6 weeks after BMT, the percentage of weight loss was significant only in the moderate GVHD group. Values are expressed as the mean ± SEM (n = 20 to 24 per group, *$P \leq .003$). (B) Clinical GVHD score of mice 6 weeks after BMT. Transplanted mice were ear tagged and evaluated from coded cages for evidence of clinical GVHD as described in the Materials and Methods. Clinical scores of allogeneic mice with mild GVHD differed significantly from scores of both syngeneic BMT recipients and allogeneic mice with moderate GVHD. Scores are expressed as mean ± SEM (n = 9 to 15 per group, *$P < .001$).

mononuclear cell infiltrate was found around both pulmonary vessels and bronchioles (Fig 2D and E). Second, an acute pneumonitis was observed involving both the interstitium and alveolar spaces (Fig 2B and F). The alveolar infiltrate was composed of macrophages, lymphocytes, epithelial cells, and scattered polymorphonuclear cells within a fibrin matrix. Both of these histopathologic patterns closely resemble the microscopic features of the nonspecific, diffuse interstitial pneumonias seen in allogeneic BMT recipients.[1,5,7,10] Other findings of diffuse alveolar injury, including alveolar hemorrhage, edema, or hyaline membranes, were not observed.

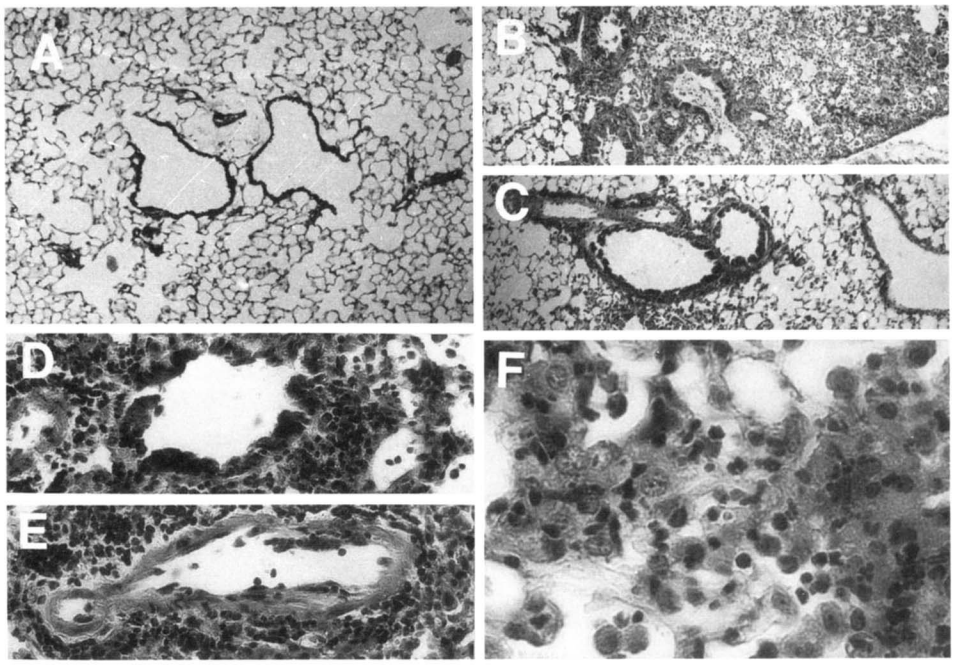

Fig 2. Pulmonary histology in CBA mice 6 weeks after syngeneic (A) and allogeneic (B through F) BMT. Mice were transplanted as described in Fig 1. Lungs were harvested and prepared for microscopic analysis as described in Materials and Methods. Interstitial inflammation is observed involving airways, vessels, and parenchyma (B) or luminal structures alone (C). Predominantly mononuclear infiltrates are observed surrounding bronchioles (D) and vessels (E). High power (×400) of interstitial and alveolar pneumonitis showing a mixed infiltrate of lymphocytes, neutrophils, and macrophages (F).

Because these two histopathologic patterns were observed independently of one another, each pattern was incorporated into a semiquantitative scoring index (Table 2). A pathology index (PI) was generated by multiplying the severity grade by the extent of involvement (range, 0 to 9) using an average of the values from both lungs. A total index score was finally calculated by adding together the PIs for pneumonitis and periluminal infiltrate (range, 0 to 18). As shown in Fig 3, the values for pneumonitis, periluminal infiltrate, and total index were significantly abnormal at 6 weeks after BMT for all animals in the allogeneic group. Surprisingly, pulmonary pathology was equivalent in allogeneic BMT recipients irrespective of GVHD severity (mild or moderate). Thus, pulmonary pathology correlated with the presence, but not with the severity, of GVHD in this model.

Table 2. Quantitation of Histopathologic Changes in the Lungs of Transplanted Mice

Score	Periluminal Infiltrates (around airways/vessels)	Pneumonitis (alveolar/interstitial)
0	No infiltrates*	No infiltrates*
1	1-3 cell diameters thick	Increased cells, only visible at high magnification (×400)
2	4-10 cell diameters thick	Easily seen cellular infiltrate or interstitial thickening
3	>10 cell diameters thick	Consolidation by inflammatory cells and interstitial thickening

The severity of histopathologic changes observed was scored using coded slides. The extent of injury was also quantitated according to the percentage of lung tissue involved (5% to 25% = 1; >25% to 50% = 2; >50% = 3). Values for total index were generated by summation of periluminal infiltrate and pneumonitis scores.

* Infiltrates are macrophages, neutrophils, lymphocytes, admixed fibrin, or edema fluid.

Potential infectious etiologies of pulmonary injury were evaluated in sentinel mice from each group (n = 5) by screening for a panel of pathologic organisms: minute virus of mice (MVM), mouse hepatitis virus (MHV), pneumonia virus of mice (PVM), reovirus 3 (REO 3), murine cytomega-

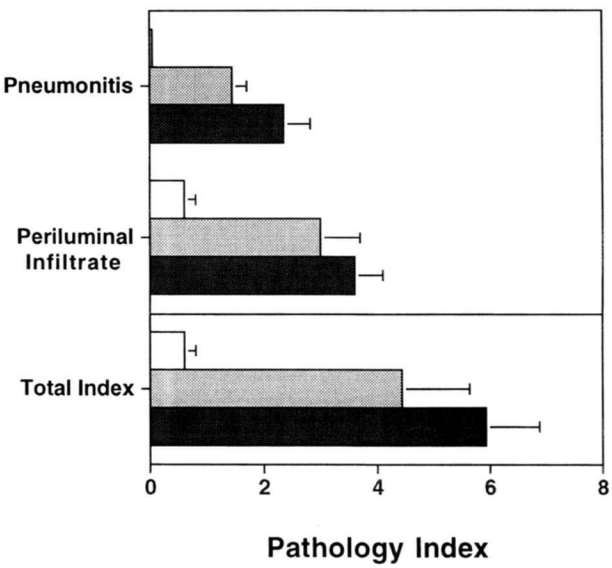

Fig 3. Semiquantitative analysis of pulmonary histopathology 6 weeks after BMT. CBA mice received allogeneic (▒, ■) or syngeneic (□) BMT, and allogeneic recipients were subsequently divided into two groups as in Fig 1. Lungs were harvested after BMT and analyzed as described in the Materials and Methods. In all categories, scores for allogeneic recipients were significantly different from those of mice receiving syngeneic BMT, $P \leq .002$; for pneumonitis in mice with mild GVHD, $P = .03$. Scores are expressed as the mean total index ± SEM (n = 9 to 12 per group).

Table 3. BAL Fluid Cellularity in Mice 6 Weeks After BMT

Group	Total	Neutrophils	Macrophages	Lymphocytes
Naive (CBA)	1.6 ± 0.3	0.01 ± 0.004	1.2 ± 0.2	0.41 ± 0.20
Syngeneic	1.9 ± 0.3	0.01 ± 0.004	1.2 ± 0.2	0.62 ± 0.09
Allogeneic (mild GVHD)	3.7 ± 1.6	0.13 ± 0.10 $P = .04$	1.8 ± 0.8	1.7 ± 0.7
Allogeneic (moderate GVHD)	6.1 ± 1.0 $P = .005$	0.43 ± 0.24 $P = .004$	2.5 ± 0.5 $P = .04$	3.1 ± 0.5 $P = .005$

CBA mice received syngeneic or allogeneic BMT, and allogeneic recipients were subsequently divided into two groups as in Fig 1. At the time of death, BAL fluid was obtained and samples were analyzed for cell count and differential as described in the Materials and Methods. Data are expressed as mean ± SEM × 10^6 cells (n = 5 to 7 per group). *P* values signify differences compared with recipients of syngeneic BMT.

lovirus (MCMV), and lymphocytic choriomeningitis virus (LCMV). Pulmonary washings were also cultured for bacteria, including *Bordetella bronchiseptic, Corynebacterium kutscheri, Klebsiella pneumoniae, K oxytoca, Mycoplasma pulmonis, Pasteurella multocida, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae,* and *Streptococcus* group B, G, and A. No pathologic organisms were identified in any mice, ruling out these infections as direct causes of lung damage. The pathology observed thus appeared to be the consequence of the minor H antigen differences between allogeneic donors and hosts.

The presence of neutrophils, endotoxin, and TNFα in BAL fluid correlates with lung histopathology 6 weeks after BMT. In an effort to analyze changes in the alveolar compartment that accompanied lung histopathology after BMT, BAL fluid was collected from all transplanted mice before fixation of lung tissue. At 2 weeks after BMT, BAL fluid cell counts were at the low range of normal for all transplanted mice, consistent with the lack of pulmonary pathology at this time (data not shown). At 6 weeks after transplantation, there was a significant increase in the total number of BAL cells harvested only from mice with moderate GVHD compared with recipients of syngeneic BMT (Table 3). Also noted in BAL fluid from this group was a fivefold increase in lymphocytes, the majority of which expressed either CD4 or CD8 (data not shown), and significant increases in macrophages and neutrophils, consistent with the mixed inflammatory alveolar infiltrates observed on histopathology (see Fig 2F). Although animals with mild GVHD showed trends toward greater cellularity in the BAL fluid, this increase was significant only for the number of neutrophils ($P = .04$), the only cell type in BAL fluid that correlated with pulmonary pathology.

The correlation of neutrophils in BAL fluid with pulmonary pathology, without evidence of acute bacterial infection, suggested that endotoxin (LPS), which is known to induce neutrophil-mediated lung injury,[24,25] might play an important role in the observed damage. We therefore analyzed the BAL fluid for the presence of endotoxin (Table 4). Syngeneic BMT recipients exhibited LPS levels that were just above the limit of detection, whereas LPS was present in significant amounts in all mice with GVHD. Similar observations were made in the serum where significantly elevated LPS levels were observed in all allogeneic BMT recipients but in no animal without GVHD. Thus, systemic and pulmonic presence of endotoxin strongly correlated with BAL neutrophils and pulmonary damage.

Because LPS has been shown to be an important stimulus for production of inflammatory mediators in animal models of GVHD,[26,27] detection of LPS in the alveolar space, an observation also reported in a recent clinical study of lung allograft rejection,[28] suggested that inflammatory cytokines might also be associated with lung injury. TNFα is a proinflammatory cytokine that has been implicated both in the pathogenesis of systemic GVHD[26,29-32] and in the development of several forms of lung toxicity.[33-36] After clinical BMT, increased serum levels of TNFα have been observed in patients with transplant-related complications, including interstitial pneumonitis.[37] We therefore analyzed BAL fluid TNFα concentrations using ELISA. As shown in Fig 4, all recipients of allogeneic BMT had significantly elevated levels of TNFα in their BAL fluid at 6 weeks after transplantation. In fact, BAL fluid TNFα levels strongly correlated with underlying pulmonary pathology ($P = .001$). TNFα levels in the BAL fluid at 2 weeks, when no pathology was apparent, remained within the normal range (10 to 20 pg/mL), just above the limits of detection of the assay. Somewhat unexpectedly, levels of IL-1β (another inflammatory cytokine) were much higher before transplantation and remained constant in transplanted animals; they actually decreased slightly in allogeneic BMT recipients by 6 weeks after transplanta-

Table 4. Endotoxin Levels in BAL Fluid and Serum of Mice 6 Weeks After BMT

Group	BAL LPS (pg/mL)	Serum LPS (pg/mL)
Naive CBA	<2	<20
Syngeneic	4 ± 1	<20
Allogeneic (mild GVHD)	11 ± 2 $P = .05$	133 ± 49 $P = .04$
Allogeneic (moderate GVHD)	21 ± 3 $P = .01$	227 ± 87 $P = .03$

CBA mice received syngeneic or allogeneic BMT, and allogeneic recipients were subsequently divided into two groups as in Fig 1. At the time of death, BAL fluid and serum were obtained and analyzed for the presence of endotoxin as described in the Materials and Methods. Endotoxin concentrations in picograms per milliliter are expressed as mean ± SEM (n = 4 to 5 per group). The limits of detection for each assay were 2 pg/mL and 20 pg/mL for BAL fluid and serum, respectively. *P* values signify differences compared with recipients of syngeneic BMT.

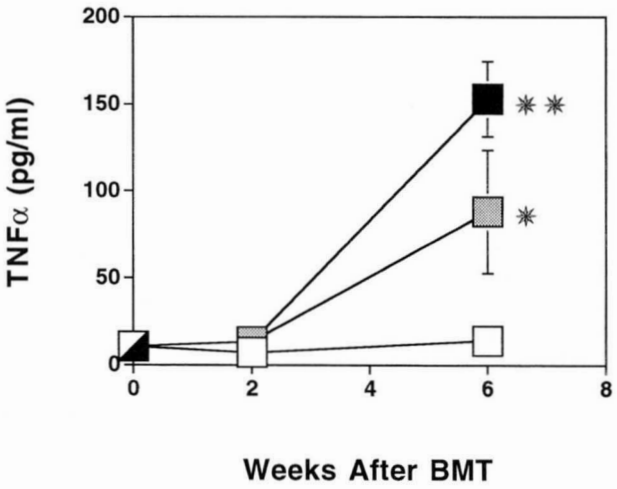

Fig 4. BAL fluid TNFα concentration after BMT. CBA mice received allogeneic (▩, ■) or syngeneic (□) BMT, and allogeneic recipients were subsequently divided into two groups as in Fig 1. BAL fluid was obtained from naive animals (◪) and at 2 and 6 weeks after BMT and analyzed for TNFα concentration as described in the Materials and Methods. Data are expressed as the mean ± SEM (n = 8 to 10 per group). *$P = .05$ and **$P < .001$ compared with syngeneic controls.

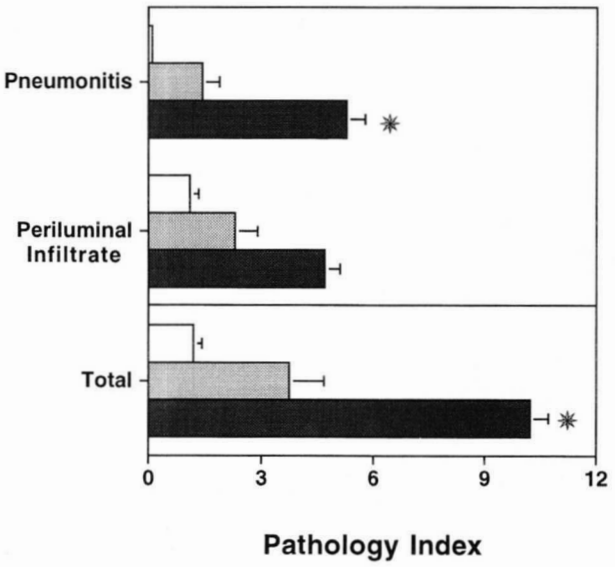

Fig 5. Effects of endotoxin challenge on lung histopathology. CBA mice received syngeneic (□) or allogeneic BMT (▩, ■), and allogeneic recipients were subsequently divided into two groups as in Fig 1. Transplanted mice were challenged with endotoxin IV (2 mg LPS) 6 weeks after BMT, and lungs were harvested and analyzed semiquantitatively as in Fig 3. Data are expressed as the mean ± SEM (n = 8 to 12 per group). *$P < .001$ compared with mice receiving no LPS (as shown in Fig 3).

tion (data not shown). Thus, with regard to these two inflammatory cytokines, pulmonary damage appeared to be specifically associated with elevated TNFα concentrations.

Exogenous endotoxin acts as a second signal to exacerbate lung injury in mice with moderate GVHD. Given the strong correlation between pulmonary disease and BAL fluid concentrations of LPS, TNFα, and neutrophils, we wanted to directly test the hypothesis that LPS triggers lung damage. To accomplish this, we challenged transplanted animals with exogenous endotoxin. A dose of 2.0 mg was chosen because 5 times this dose produced transient inactivity but no mortality in naive CBA mice in pilot experiments (data not shown). LPS was dissolved in normal saline and administered intravenously to mice 6 weeks after transplantation; control mice received saline alone. None of 12 syngeneic BMT mice and 0 of 11 mice with mild GVHD died, whereas 7 of 21 mice with moderate GVHD died, a highly significant difference ($P = .01$). Surviving mice were analyzed 18 to 36 hours after injection for BAL fluid changes and lung histopathology. When compared with animals receiving no LPS (Table 3), LPS injection increased the total number of neutrophils in the BAL fluid of mice with moderate GVHD (Table 5). Neither the number of lymphocytes nor the number of macrophages was increased compared with saline controls, although the activation status of these cells was enhanced, as judged by a significant increase in the number of Mac 1^+ cells ($13.0 \pm 2.0 \times 10^5$ *v* $3.7 \pm 1.4 \times 10^5$, $P < .01$). No significant changes were noted in BAL fluid of animals with mild GVHD or in syngeneic BMT recipients. When lungs were scored for histopathology, LPS administration significantly amplified the severity of lung injury, particularly pneumonitis, only in animals with moderate GVHD compared with saline-treated controls (Fig 5, $P < .004$). These pathologic changes were again associated with dramatic increases in TNFα in the BAL fluid of affected animals (Fig 6, $P < .01$). In addition, alveolar hemorrhage, which was not observed in any other setting, was seen in 4 of 12 animals with moderate GVHD receiving LPS ($P < .001$). The degree of hemorrhage varied from extensive alveolar flooding (1 of 4) to moderate numbers of red blood cells within pneumoni-

Table 5. Effects of Endotoxin Challenge on BAL Fluid Cellularity

Group	Total	Neutrophils	Macrophages	Lymphocytes
Syngeneic	0.75 ± 0.24	0.01 ± 0.001	0.52 ± 0.20	0.19 ± 0.04
Allogeneic (mild GVHD)	3.1 ± 0.8	0.37 ± 0.14	1.2 ± 0.3	1.4 ± 0.4
Allogeneic (moderate GVHD)	7.4 ± 0.9	2.6 ± 0.4	2.7 ± 0.4	2.0 ± 0.2
		$P = .006$		

CBA mice received syngeneic or allogeneic BMT, and allogeneic recipients were subsequently divided into two groups as in Fig 1. Transplanted mice were challenged with endotoxin IV (2 mg LPS) 6 weeks after BMT. At the time of death, BAL fluid was obtained and samples were analyzed for cell count and differential as in Table 3. Data are expressed as mean ± SEM × 10^6 cells (n = 5 to 9 per group). The *P* value signifies the difference compared with mice receiving no LPS (as shown in Table 3).

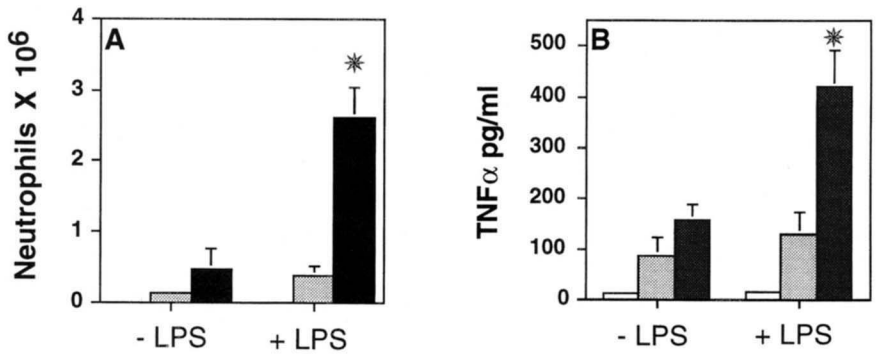

Fig 6. Effects of endotoxin challenge on BAL fluid contents. CBA mice received syngeneic (□) or allogeneic BMT (▩, ■), and allogeneic recipients were subsequently divided into two groups as in Fig 1. Transplanted mice were challenged with endotoxin IV (2 mg LPS) 6 weeks after BMT, and BAL fluid was harvested and analyzed for neutrophils (A) and TNFα concentration (B). Data are expressed as the mean ± SEM (n = 5 to 12 per group). *$P \leq .01$ compared with saline-treated controls.

tic areas (3 of 4). Endotoxin challenge therefore leads to enhanced mortality and caused both quantitative and qualitative changes in pulmonary pathology only in mice with moderate GVHD. These findings strongly suggest that mice with mild GVHD and those receiving syngeneic BMT could effectively detoxify the LPS challenge and thus prevent further lung damage, whereas animals with moderate GVHD could not.

To further evaluate the importance of GVHD severity to ineffective endotoxin clearance, we analyzed hepatic histology in a limited number of randomly chosen transplanted mice. The liver represents the body's principal mechanism for detoxification of LPS (Kupffer cells that line the hepatic sinusoids form approximately 85% of the body's reticuloendothelial mass) and the subsequent clearance of LPS from the circulation.[38,39] Using detailed, semiquantitative criteria of hepatic histopathology in GVHD as previously published,[16] we observed that hepatocellular damage shown by the presence of pan-lobar necrosis, microabscesses, and increased numbers of acidophil bodies and mitotic figures was present but did not differ between mild and moderate GVHD groups in unchallenged animals (data not shown). By contrast, administration of exogenous LPS increased this damage in the moderate GVHD group compared with LPS challenged mice with mild GVHD (3.8 ± 1.5 *v* 1.0 ± 0.8, $P < .04$, n = 4 to 6 per group). These data confirm that the severity of GVHD is critical to the ability of hosts to withstand LPS challenge and suggest an important role for the liver in protecting the lungs of transplanted animals from LPS-mediated damage.

DISCUSSION

We hypothesized that the lung may represent a target organ of GVHD and that endotoxin and TNFα play important roles in the pathogenesis of IPS after allogeneic BMT. Our data support this hypothesis because, first, we show significant pulmonary toxicity 6 weeks after BMT in all mice developing GVHD to minor H antigens (Fig 3). The microscopic features of this damage are consistent with previous experimental reports and with histologic changes found in association with clinical IPS[1,5,7,17,40,41] and included mononuclear inflammatory infiltrates within alveoli and interstitial spaces and surrounding bronchioles and vessels (Fig 2). Second, this histopathology was associated with significant changes in BAL fluid composition, including increased neutrophils, endotoxin, and TNFα levels (Fig 4 and Tables 3 and 4). Third, challenging transplanted mice with endotoxin exacerbated pulmonary damage only in animals with moderate GVHD (>10% weight loss), which was again reflected in BAL fluid changes and produced a qualitative histopathologic change (alveolar hemorrhage) in a significant percentage of this group (Figs 5 and 6).

The role of alloreactivity in the generation of IPS is controversial. For example, lung injury was not noted in an unirradiated animal model of systemic GVHD.[42] By contrast, a subsequent animal study using an irradiated BMT model of GVHD to MHC antigens showed two forms of lung injury and provided the first data implicating TNFα as a mediator of pulmonary damage.[41] More recently, the development of interstitial pneumonitis and lymphocytic bronchiolitis/bronchitis similar to the histopathology seen in lung allograft rejection was observed over a 3-week period in an unirradiated GVHD model to MHC differences in rats.[40] We have recently observed pulmonary pathology in the murine model of GVHD to minor histocompatibility antigens studied here and found that mortality was associated with lung injury and correlated with both the amount of total body irradiation administered for pretransplantation conditioning and the dose of donor T cells in the marrow inoculum.[17] The current study extends our previous observations and provides additional information regarding the pathogenesis of IPS after allogeneic BMT.

Possible mechanisms of noninfectious lung injury after BMT include cytotoxic effects of drugs or irradiation used in pretransplantation conditioning, cell-mediated tissue damage, and the secretion of inflammatory cytokines. Recently, the importance of inflammatory cytokines as critical effector molecules of GVHD has been noted by several groups.[18,26,31,43] Pertinent to our hypothesis, inflammatory cytokines, in particular TNFα, have also been identified in several forms of lung damage, including infectious pneumonia, adult respiratory distress syndrome, lung allograft rejection, and pneumo-

nitis secondary to toxin exposures.[33,35,44-46] In addition to its direct toxic effects, TNFα increases the expression of specific adherence proteins on pulmonary endothelium and may stimulate the release of IL-8 (a potent neutrophil chemotactic factor) from pulmonary epithelial cells and fibroblasts.[34] Currently, the lung is thought to be the site of complex cytokine networks, the proper balance of which allows for infectious surveillance and maintenance of structural integrity, whereas its dysregulation can result in tissue injury and scarring.[33] From this perspective, lung inflammation is the result of a multifaceted immune response involving the interaction between inflammatory cells (eg, macrophages, monocytes, and neutrophils), soluble cytokines, and lung endothelium.[33,34] Both TNFα and LPS are thought to have an integral role in this process.[46,47]

Although our data support the hypothesis that endotoxin and TNFα are important mediators in the development of IPS in this experimental model, their precise role in the evolution of lung damage remains unresolved. Interestingly, a recent study that proposed a mechanism for endothelial damage after BMT showed that endotoxin enhances (via TNFα) endothelial cell apoptosis induced by irradiation.[48] In our study, the two major microscopic patterns of lung injury identified were parenchymal pneumonitis and perivascular/bronchial infiltrates. Although at times contiguous, the lesions were predominantly discrete and involved varying amounts of lung tissue. It is possible that early endothelial injury caused by irradiation may be self-limited in the setting of syngeneic BMT but subsequently enhanced by a systemic and/ or targeted graft-versus-host reaction (driven by endotoxin and TNFα) after allogeneic BMT. With progressive damage, a breach in the endothelial barrier could allow for the passage of inflammatory cells and mediators first into the adjacent periluminal areas and eventually into the pulmonary parenchyma.

The results of the endotoxin challenge experiments indirectly support this theory and further suggest that (1) parenchymal pneumonitis represents a response to LPS because only this inflammatory process (and not the periluminal infiltrates) increased after LPS injection and (2) endotoxin can trigger the release of cytotoxic amounts of inflammatory mediators and serve as a potent stimulus for the recruitment of neutrophils to the site of acute inflammation. Although endotoxin challenge had no effect in animals when GVHD was absent or mild, enhanced lung injury, along with increased neutrophils and TNFα in BAL fluid, was noted after LPS challenge in animals with more extensive (moderate) GVHD; in fact, a unique pathologic finding (alveolar hemorrhage) was observed exclusively in this group. Diffuse alveolar hemorrhage (DAH) has been reported in murine GVHD models[41] and can occur as a terminal complication associated with clinical GVHD[7,49] or after autologous BMT. The occasional responsiveness of DAH to corticosteroids in the latter scenario also supports a possible effector role for inflammatory cytokines.[50,51]

Two alternative explanations, neither mutually exclusive of the other, may also account for the above findings. First, macrophages in more severely affected animals in which the graft-versus-host reaction is active and ongoing may remain primed to respond to LPS with the production of cytopathic amounts of inflammatory mediators as shown by Nestel et al[26] in another model of acute GVHD. Second, the ability of systemic endotoxin to reach the alveolar space may be directly related to the consequences of GVHD in other target organs. From the perspective of pulmonary damage, the liver is probably critical in this regard. It is pivotally located immediately downstream (via the splanchnic circulation) of the intestinal reservoir of gram-negative bacteria and their toxic byproducts. When confronted with a sudden endotoxin surge, liver macrophages produce and export inflammatory cytokines. If the endotoxin load surpasses the hepatic capacity for its clearance, both inflammatory cytokines and unprocessed endotoxin will spill over into the systemic circulation. Several experimental studies have shown that the presence of pre-existing hepatic injury decreases the threshold at which the liver can effectively neutralize endotoxin.[52-54] In the setting of acute GVHD, the endotoxin surge arises from increased LPS translocation across a damaged intestinal mucosa. In this scenario, underlying damage as a consequence of hepatic GVHD could then serve to decrease the liver's capacity for LPS uptake and clearance. As seen in our study, animals with no or mild GVHD were able to effectively detoxify the administration of exogenous endotoxin and protect their lungs from further damage. By contrast, the same amount of endotoxin could not be neutralized by animals with moderate GVHD, as shown by the significant increase in hepatocellular damage, lung injury, and mortality observed in this group. LPS could therefore remain in the systemic circulation for prolonged periods, triggering pulmonary mononuclear cell populations to secrete additional inflammatory cytokines and ultimately enhancing tissue damage. These experiments thus support the notion of a gut-liver-lung axis during GVHD pathophysiology[39,55] and would suggest that any process or combination of events that allows large amounts of endotoxin into the pulmonary circulation and/or TNFα into the alveolar space could promote the development of lung injury.

In conclusion, we have shown that noninfectious lung injury after BMT occurred only in the setting of GVHD induced by minor H antigenic differences and that TNFα and endotoxin are associated with this process. Although the severity of GVHD did not correlate with the intensity of lung injury in this model, the inability of more severely affected animals to neutralize an endotoxin challenge led us to hypothesize that the liver may have a pivotal role in the generation of pulmonary inflammation during GVHD. Because both LPS and TNFα are also considered to have critical roles in the pathogenesis of GVHD, our data suggest that IPS after allogeneic BMT shares, at least in part, a common immunopathophysiologic pathway with GVHD. Experiments are currently in progress to define the precise relationships between these disease entities and evaluate the ability of specific cytokine inhibitors to prevent or reverse pulmonary damage.

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