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

By Kenneth R. Cooke, Lester Kobzik, Thomas R. Martin, Joanne Brewer, John Delmonte Jr, James M. Crawford, and James L.M. Ferrara

Idiopathic pneumonia syndrome (IPS) refers to diffuse, non-infectious 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 pneumonia 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.

There are 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. This form of noninfectious posttransplantation lung injury has been named idiopathic pneumonia syndrome (IPS) by a recent National Institutes of Health panel. 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, bronchoalveolar 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 edema or hemorrhage, and hyaline membranes. 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%.

In 1973, Neiman et al 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. In 1978, Beschorner et al 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. A linear correlation between the onset of acute GVHD and respiratory symptoms was also cited, an association that was verified in some reports but not in others in which IP was also seen after autologous and syngeneic BMT. 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.

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 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 histo-

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Submitted December 18, 1995; accepted June 14, 1996.

Supported by National Institutes of Health Grants No. HL55162, AI 30018, CA 39542, and DK3512. J.L.M.F. is a scholar of the Leukemia Society of America.

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0006-4971/96/8808-0028$3.00/0
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-2d) and B10.BR (H-2b) 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 × 10^6 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 (137Cs source) delivered in two fractions separated by 9 hours to reduce gastrointestinal toxicity. This dose of irradiation has been shown not to cause histologically detectable pulmonary injury in normal CBA mice.1e 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 0.8-mL aliquot of 0.8% (vol/vol) saline (PBS) containing 0.6 mM 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 (pH 7.4, <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 × 10^6 cells were washed two additional times with 2% fetal bovine serum (FBS) in PBS. Fc receptors were blocked with monoclonal antibody (MoAb) 2.4G2 at 4°C for 15 to 20 minutes. Cells were then incubated at 4°C for 30 minutes with fluorescein isothiocyanate– or R-phycocerythrin–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.

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-fixed paraffin-embedded sections were cut to 5-μm thickness sections, dehydrated with ethanol and eosin (H & E) for histologic examination. Slides were coded without reference to mouse type or prior treatment status and examined by a pathologist (J.M.C.). Liver tissue pathology was scored semiquantitatively using a detailed system previously described.20,23

Table 1. Assessment of Clinical GVHD in Transplanted Animals

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight loss</td>
<td>&lt;10%</td>
<td>&gt;10% to &lt;25%</td>
<td>&gt;25%</td>
</tr>
<tr>
<td>Postures</td>
<td>Normal</td>
<td>Hunching noted only at rest</td>
<td>Severe hunching impairs movement</td>
</tr>
<tr>
<td>Activity</td>
<td>Normal</td>
<td>Mild to moderately decreased</td>
<td>Stationary unless stimulated</td>
</tr>
<tr>
<td>Fur texture</td>
<td>Normal</td>
<td>Mild to moderate ruffling</td>
<td>Severe ruffling/poor grooming</td>
</tr>
<tr>
<td>Skin integrity</td>
<td>Normal</td>
<td>Scaling of paws/tail</td>
<td>Obvious areas of denuded skin</td>
</tr>
</tbody>
</table>

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, 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.

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 antимurine 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 receiving 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.BL) 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.2 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 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.3,4,7,10 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

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

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-
loivirus (MCMV), and lymphocytic choriomeningitis virus (LCMV). Pulmonary washings were also cultured for bacteria, including Bordetella bronchiseptica, Corynebacterium kutscheri, Klebsiella pneumoniae, 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, 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, detection of LPS in the alveolar space, an observation also reported in a recent clinical study of lung allograft rejection, 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 and in the development of several forms of lung toxicity. After clinical BMT, increased serum levels of TNFα have been observed in patients with transplant-related complications, including interstitial pneumonitis. 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 transplantation.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive (CBA)</td>
<td>1.6 ± 0.3</td>
<td>0.01 ± 0.004</td>
<td>1.2 ± 0.2</td>
<td>0.41 ± 0.20</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>1.9 ± 0.3</td>
<td>0.01 ± 0.004</td>
<td>1.2 ± 0.2</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td>Allogeneic (mild GVHD)</td>
<td>3.7 ± 1.6</td>
<td>0.13 ± 0.10</td>
<td>1.8 ± 0.8</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>Allogeneic (moderate GVHD)</td>
<td>6.1 ± 1.0</td>
<td>0.43 ± 0.24</td>
<td>2.5 ± 0.5</td>
<td>3.1 ± 0.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Group</th>
<th>BAL LPS (pg/mL)</th>
<th>Serum LPS (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive CBA</td>
<td>&lt;2</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>≤ 1</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Allogeneic (mild GVHD)</td>
<td>11 ± 2</td>
<td>133 ± 49</td>
</tr>
<tr>
<td></td>
<td>P = .05</td>
<td>P = .04</td>
</tr>
<tr>
<td>Allogeneic (moderate GVHD)</td>
<td>21 ± 3</td>
<td>227 ± 87</td>
</tr>
<tr>
<td></td>
<td>P = .01</td>
<td>P = .03</td>
</tr>
</tbody>
</table>

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.
EXPERIMENTAL IPS, GVHD, AND ENDOTOXIN

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 = 6 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).

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 ± 2.0 × 103 vs 3.7 ± 1.4 × 103, 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngeneic</td>
<td>0.75 ± 0.24</td>
<td>0.01 ± 0.001</td>
<td>0.52 ± 0.20</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Allogeneic (mild GVHD)</td>
<td>3.1 ± 0.8</td>
<td>0.37 ± 0.14</td>
<td>1.2 ± 0.3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Allogeneic (moderate GVHD)</td>
<td>7.4 ± 0.9</td>
<td>2.6 ± 0.4</td>
<td>2.7 ± 0.4</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

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 × 10⁶ 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 < .01 compared with saline-treated controls.

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 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. 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. More recently, the development of interstitial pneumonitis and lymphocytic bronchiolitis/brazilitis 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. 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. 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 cytokotoxic 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. 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. 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. 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. From this perspective, lung inflammation is the result of a multifaceted immune response involving the interaction between inflammatory cells (e.g., macrophages, monocytes, and neutrophils), soluble cytokines, and lung endothelium. Both TNFα and LPS are thought to have an integral role in this process.

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. 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 cytokotoxic 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 and can occur as a terminal complication associated with clinical GVHD or after autologous BMT. The occasional responsiveness of DAH to corticosteroids in the latter scenario also supports a possible effector role for inflammatory cytokines.

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 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. 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 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.

ACKNOWLEDGMENT

The authors thank Drs. W. Krenge, L. Pan, and P. Martin for their helpful and stimulating discussions; E. Moore, S. Hartnett, and
REFERENCES

1. Clark J, Hansen J, Hertz M, Parkman R, Jensen L, Peavy H: 
Idiopathic pneumonia syndrome after bone marrow transplantation. 
Am Rev Respir Dis 147:1601, 1993

2. Crawford S, Hackman R: Clinical course of idiopathic pneu-
monia after bone marrow transplantation. Am Rev Respir Dis 
147:1393, 1993

3. Weiner RS, Mortimer MB, Gale RP, Gluckman E, Kay HEM, 
Kolb JJ, Hartz AJ, Rimm AA. Interstitial pneumonitis after bone 

4. Quacke K: The lung as a critical organ in marrow transplanta-
tion. Bone Marrow Transplant 14:519, 1994

5. Crawford SC, Hackman RC: Clinical presentation, pathology 
and outcome of idiopathic pneumonia after marrow transplantation. 
Am Rev Respir Dis 141:A48, 1990 (abstr)

6. Perreault C, Cousineau S, Giovanni D, Gyger M, Nepveu F, 
Bioleau J, Bonny Y, Lacombe M, Lavallee R: Lymphoid interstitial 
pneumonia after allogeneic bone marrow transplant. Cancer 55:1, 1985

7. Sioane J, Depleldge M, Bowles R, Morgenstern G, Trickey B, 
Dady P: Histopathology of the lung after bone marrow transplanta-

KG, Stob R, Buckner CD, Cliff RA, Fefer L, Gluckberg H, Thomas 
ED: Interstitial pneumonia and cytomegalovirus infection as compli-
cations of human marrow transplantation. Transplantation 15:478, 
1973

and the risks for idiopathic pneumonia after marrow transplanta-
tion for severe aplastic anemia. Bone Marrow Transplant 12:225, 
1993

Lymphocytic bronchitis associated with graft versus host disease in 
recipients of bone marrow transplants. N Engl J Med 299:1030, 
1978

11. Bortin M, Ringden O, Horowitz M, Rozman C, Weiner R, 
Rimm A: Temporal relationships between the major complication 
of bone marrow transplantation for leukemia. Bone Marrow 
Transplant 4:339, 1988

12. Appelbaum FR, Meyers JD, Fefer A, Floumoy N, Cheever 
MA, Greenberg PD, Hackman R, Thomas ED: Nonbacterial nonfun-
damental pneumonia following marrow transplantation in 100 identi-
cal twins. Transplantation 33:265, 1982

13. Pecego R, Hill R, Appelbaum FR, Amos D, Buckner CD, 
Fefer A, Thomas ED: Intersitial pneumonitis following autologous 
bone marrow transplantation. Transplantation 42:516, 1981

B: Pulmonary pathology in bone marrow transplant recipients. Lab 
Invest 46:3, 1982

bone marrow transplantation across minor histocompatibility barriers 
in mice. Prevention by removing mature T cells from marrow. J Exp 

16. Blatter DD, Crawford JM, Ferrara JLM: Nuclear magnetic 
resonance of hepatic graft-versus-host disease in mice. Transplantation 
50:1011, 1990

17. Down JD, Mauch P, Warhol M, Neben S, Ferrara JLM: The 
effect of donor T lymphocytes and total-body irradiation on hemo-
poietic engraftment and pulmonary toxicity following experimental 
allogeneic bone marrow transplantation. Transplantation 54:802, 
1992

18. Abhyankar S, Gilliland DG, Ferrara JLM: Interleukin 1 is a 
clinical effector molecule during cytokine dysregulation in graft-
versus-host disease to minor histocompatibility antigens. Transplan-
tation 53:1518, 1993

19. Blazar BR, Taylor PA, Snover DC, Sehgal SN, Vallera DA: 
Murine recipients of fully mismatched donor marrow are protected 
from lethal graft-versus-host disease by the in vivo administration 
of rapamycin but develop an autoimmune-like syndrome. J Immunol 
151:5726, 1993

20. Blazar BR, Taylor PA, Panosksaltis-Mortari A, Gray G, 
Vallera DA: Coblockade of the LFA1:ICAM and CD28/CTLA4:B7 
pathways is a highly effective means of preventing acute lethal 
graft-versus-host disease induced by fully major histocompatibility 

21. Fontaine P, Perreault C: Diagnosis of graft-versus-host disease 
in mice transplanted across minor histocompatibility barriers. 
Transplantation 49:1177, 1990

22. Unkeless JC: Characterization of a monoclonal antibody di-
rected against mouse macrophage and lymphocyte Fc receptors. 

23. Shulman RM, Sharma P, Amos D, Fenster LF, McDonal GB: 
A coded histologic study of hepatic graft-versus-host disease after 
human bone marrow transplantation. Hepatology 8:463, 1988

Respir Dis 133:913, 1986

25. Strieer RM: Endothelial cell gene expression of a neutrophil 
chemotactic factor by TNFcr, LPS and IL-1. J Science 243:1467, 
1989

priming and lipopolysaccharide-triggered release of tumor necrosis 
factor alpha during graft-versus-host disease. J Exp Med 175:405, 
1992

JLM: Polarized type 2 alloreactive CD4+ and CD8+ donor T cells 
fail to induce experimental acute graft-versus-host disease. J Immunol 
155:585, 1995

28. Lambert SB, Frazier-Jessen MR, VanStedum S, Huhn EL, 
Filkins J, Garrity ER Jr, Kovacs EJ: Bronchoalveolar lavage fluid 
endotoxin elevation in human single lung transplant recipients during 
rejection. Transplant Immunol 3:61, 1995

29. Eller R, Kolb HJ, Hintermeier-Kaabe R, Mittermuller J, 
Thierfelde S, Kaul M, Wilmanns W: The role of tumor necrosis 
factor alpha in acute graft-versus-host disease and complications 
following allogeneic bone marrow transplantation. Transplant Proc 
25:1234, 1993

30. Eller R, Kolb HJ, Mittermuller J, Kaul M, Ledderose G, 
Duell T, Sebeer B, Scheleuning M, Hintermeier-Kaabe R, Ertl B, 
Kempeni J, Wilmanns W: Modulation of acute graft-versus-host disease by tumor necrosis 
factor (TNFa) release in the course of pretransplant conditioning: Role of conditioning regimens and prophylactic application of a monoclonal antibody neutralizing human TNFcr (MAK 195F). Blood 86:890, 1995


33. Kunkel SL, Strieer RM: Cytokine networking in lung inflam-

34. Kelley J: Cytokines of the lung. Am Rev Respir Dis 141:765, 
1990


36. Cooke ET AL
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An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin

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