Cytokine Dysregulation and Acute Graft-Versus-Host Disease

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It has been 25 years since Billingham defined the essential elements of graft-versus-host disease (GVHD) in a Harvey Lecture. The next 25 years of intensive study of GVHD confirmed that acute GVHD is caused by T lymphocytes contained in the donor graft that recognize antigenic disparities between donor and recipient. Efforts to control GVHD have therefore been directed against the donor-derived T cell. Thus, cyclosporine ameliorates GVHD by inhibiting the increased expression of interleukin-2 (IL-2) and IL-2 receptor (IL-2R) by T lymphocytes during activation. Methotrexate kills T lymphocytes that are proliferating in response to stimulation by recipient antigens. Depleting the graft of T cells by a variety of techniques can reduce GVHD by limiting the number of alloreactive cells in the infused marrow. However, both pharmacologic prophylaxis of GVHD and T-cell depletion have substantial pitfalls. Cyclosporine, methotrexate, corticosteroids, and other drugs are not completely effective in controlling activation. Methotrexate kills T lymphocytes that are proliferating. T-cell depletion is more effective than pharmacologic prophylaxis at preventing GVHD, but is associated with a higher risk of graft rejection as well as loss of the graft-versus-leukemia (GVL) effect, an important adjunct in the control of leukemia.

An ideal prophylactic regimen would reduce the organ damage associated with GVHD and not impair hematopoietic engraftment and the GVL effect. It is likely that the development of such a regimen will require new insights into the pathophysiology of GVHD. Data are accumulating that mechanisms other than direct T-cell-mediated cytotoxicity may generate tissue damage associated with GVHD. Indeed, several convergent lines of evidence implicate a network of inflammatory cytokines as primary mediators of acute GVHD. We hypothesize that the activation of T cells is but one critical step in a three-step process of GVHD pathophysiology. Most of the clinical manifestations of GVHD may, in fact, be due to dysregulated production of inflammatory cytokines secreted by cells other than T cells. This cytokine network may be the final common pathway of the tissue damage associated with acute GVHD, and the rapid onset of severe acute GVHD may be considered a “cytokine storm.” If correct, this hypothesis suggests new approaches to the management of GVHD beyond T-cell elimination or paralysis.

Acute GVHD as a Three-Step Process

We envisage acute GVHD as a three-step process (Fig 1). First, the expression of HLA and leukocyte adhesion molecules on target tissues such as skin, intestinal mucosa, and liver is increased by cytokines released as a result of conditioning regimen toxicity, infections, and possibly the underlying disease. In syngeneic or autologous transplants, or after T-cell depletion of allogeneic marrow, this production of inflammatory cytokines is self-limited and resolves within 7 to 10 days. However, in allogeneic marrow grafting, a second step occurs when mature donor T cells recognize histocompatibility antigens in the host and become activated. T-cell recognition of histocompatibility antigens and adhesion to targets is facilitated by consequences of the first step, ie, the cytokine-induced increases in host cell surface receptors in these tissues and the activation of T cells by cytokines. The activated donor T cells then proliferate, express IL-2R, and secrete IL-2. Third, IL-2 activates newly engrafted mononuclear cells from the donated marrow to secrete more inflammatory cytokines, such as IL-1, tumor necrosis factor (TNFα), and interferon-γ (IFNγ). The resulting inflammatory response results in additional release of cytokines and amplifies local organ injury. Thus, a network of soluble cytokines form a critical link between each of the three steps and may be responsible for the bulk of target organ damage. The evidence for cytokine dysregulation as a primary mechanism of acute GVHD is summarized below.

Step One: The Role of Host Tissues in the Development of Acute GVHD

Most of the attention in GVHD-related research has been directed at donor T cells. An understanding of acute GVHD must also take into account the milieu in which the T cells function and the effects of the conditioning regimen, infection, transfusions, prior therapy, and the underlying disease on endothelial and epithelial cells. Several analyses of large numbers of transplants indicate that the risk of GVHD increases with advanced-stage leukemia, certain intensive conditioning regimens, and perhaps with certain viral infections. As discussed above, one explanation for these associations is that epithelial and endothelial injury stimulates the release of inflammatory cytokines, which increases the expression of cell-surface adhesion molecules (eg, integrins) and histocompatibility proteins. For example, endotoxin from the gut may leak through the bowel wall and stimulate endothelial cells and/or macrophages to produce cytokines such as IL-1, TNFα, IL-6, and natural killer cell stimulatory factor (NKSF) even in the absence of clinical sepsis. Endotoxin from gram-negative rods on the skin may stimulate keratinocytes, macrophages and endothelial cells to produce cytokines in the dermis and epidermis.
This response includes the autocrine production of IL-2 and IFNγ, the conditioning regimen cytokines such as IL-6, IL-8, and NKSF may further amplify the injury which in turn activate mononuclear cell effectors from donor marrow. In step 3, additional production of inflammatory cytokines by these donor mononuclear cells results in direct tissue injury. Endotoxin released by gram-negative bacteria as well as production of other cytokines such as IL-6, IL-8, and NKSF may further amplify the injury and recruit additional effectors.

The ability of these cells to produce inflammatory mediators in response to injury is well established.7 Holler et al in Munich have conclusively demonstrated the importance of TNFα, which is released from host tissues during this first step of acute GVHD.8 Initially, they reported that serum levels of TNFα increased approximately twofold in patients with grade II acute GVHD and increased almost threefold in patients with more severe acute GVHD. In addition, the maximal TNFα serum level peaked shortly after completion of the conditioning regimen, and an average of 25 days before the diagnosis of acute GVHD. Further clinical data from this group showed that the kinetics of elevated serum TNFα levels are even more predictive than the maximal TNFα level observed with respect to transplant-related complications and overall survival.9 Greater than 90% of patients who showed abnormal TNFα levels during the transplant conditioning developed significant acute GVHD and had less than 30% long-term survival. By contrast, patients who first developed increased TNFα levels between day +1 and day +90 had a 50% survival, and patients who had normal TNFα levels until at least day +90 had an 80% survival. Recent experiments in a murine model showed that the single injection of an anti-TNFα antibody before recipient irradiation and transplantation of allogeneic marrow significantly reduced subsequent GVHD mortality from 58% to 17%.9 Taken together, these data argue strongly for an early role of inflammatory cytokines released from host tissues that enhance the presentation of host alloantigens to donor T cells.

**STEP TWO: THE ROLE OF T CELLS IN ACUTE GVHD**

The importance of T cells in GVHD is underscored by numerous observations demonstrating that, below a threshold number of T cells, the risk of GVHD diminishes with a further decline in the number of marrow T lymphocytes.10,11 Nevertheless, evidence that acute GVHD is directly mediated by cytotoxic T lymphocytes is meager. Typically, pathologic specimens of skin, liver, or gut involved with acute GVHD are striking for the disproportion between tissue damage and the intensity of the lymphoid infiltrate in the organ. In addition, controlled trials of antithymocyte globulin and immunotoxins have been disappointing in the therapy of acute GVHD.12,13 IL-2 is critical for the activation and proliferation of T lymphocytes; it has been considered, therefore, to play an important role in acute GVHD. However, the role of IL-2 as an effector molecule of GVHD-induced damage has been difficult to establish. Serum IL-2 levels are not increased in acute GVHD, and while increases in IL-2R levels have been observed,14 the significance of this observation is uncertain because many cell types have IL-2 receptors. Anti–IL-2R monoclonal antibodies provide only transient benefit in steroid-resistant GVHD and their mechanism of action is unclear.15 Cyclosporine is incompletely effective in preventing acute GVHD, suggesting either that the inhibition of IL-2 production is incomplete or that other mechanisms are responsible for GVHD. Furthermore, IL-2 can be administered safely after T-cell depleted or allogeneous marrow grafting without causing or accelerating GVHD.16,17 The only setting in which IL-2 can be directly implicated in causing a “GVH-like” syndrome is when large doses of IL-2 are administered with lymphokine-activated killer (LAK) cells in the therapy of certain malignancies.18 Even then, much of the IL-2 toxicity is probably mediated through the induction of other inflammatory cytokines.19 Arguments favoring an indirect mechanism for IL-2 toxicity include: (1) the induction of TNF and IL-1 synthesis by peripheral blood mononuclear cells incubated in IL-220; (2) the extensive overlap between IL-2, IL-1, and TNF toxicities in both experimental and clinical settings21,22; and (3) the inability of IL-2 to act directly on a variety of cell types in vitro, even when those same cell types are clearly affected by administration of IL-2 in vivo.23 Corticosteroids, which are often an effective therapy for acute GVHD, are typically thought to reduce T-cell numbers; but corticosteroids also diminish the expression and production of inflammatory cytokines, particularly IL-1 and TNFα, independent of their effect on T-cell viability. Thus, while the importance of T cells in acute GVHD is unquestioned, their primary role may reflect cytokine production and recruitment of other effectors, rather than direct cell-mediated cytotoxicity.

**STEP THREE: THE ROLE OF CYTOKINES AS EFFECTORS**

Murine models have provided strong evidence of the link between excessive or dysregulated cytokine production and clinical GVHD. TNFα is well established as a cytokine that
causes organ damage in experimental acute GVHD. Mice receiving transplants of allogeneic bone marrow plus additional T cells develop severe skin, and lung lesions that are associated with high levels of TNFα messenger RNA (mRNA) in these tissues. The effects on target organs were ameliorated by infusing anti-TNFα antibodies. More recent evidence demonstrates TNFα mRNA in the skin of mice developing GVHD after transplants across minor histocompatibility barriers. Studies of TNFα in blood mononuclear cells. Recent data from Dickinson et al further implicated inflammatory cytokines in the pathogenesis of GVHD by showing an association between increased TNFα and IFNγ synthesis in a skin explant model and the development of acute GVHD. Anti-TNFα monoclonal antibodies are capable of temporarily abrogating steroid-resistant acute GVHD in human GVHD, but the transience of the response suggested that the underlying pathophysiology had not been entirely interrupted. Early clinical studies indicate that when TNFα production is inhibited with pentoxifylline or the combination of pentoxifylline, ciprofloxacin, and prednisone, clinical GVHD as well as other regimen-related toxicity was ameliorated.

Recent experimental data demonstrate that IL-1, a central mediator of inflammation, plays an important role in GVHD. IL-1 mRNA increases in the skin of mice after transplant only if the skin is involved with GVHD. A similar increase in mononuclear cell IL-1 mRNA has been observed during clinical acute GVHD. If IL-1 is a central mediator of GVHD, specific inhibition of IL-1 action should result in a reduction or elimination of GVHD. IL-1 receptor antagonist (IL-1ra) is a specific, competitive inhibitor of both IL-1α and IL-1β. When administered to mice either for prophylaxis or therapy of GVHD, mortality from GVHD was strikingly diminished. The role of IL-1 may also be indirectly inferred from the high-dose γ-globulin trials of GVHD prophylaxis. High-dose γ-globulin appears to reduce the incidence of acute GVHD. A relevant in vitro observation is that IgG bound to mononuclear cell Fe receptors increases IL-1α production preferentially over IL-1α or IL-1β (W. Arend, personal communication), suggesting that a similar mechanism might occur in vivo. Granulocyte-macrophage colony-stimulating factor (GM-CSF) seems to potentiate this preferential production of IL-1ra, and ongoing studies may determine whether GM-CSF can reduce the incidence of clinical GVHD.

Cytokines cause a large number of endothelial cell changes that might enhance the tissue destruction associated with acute GVHD. In addition to increased expression of histocompatibility antigens and leukocyte adhesion molecules, inflammatory cytokines may directly or indirectly also increase prostacyclin production, nitric oxide (endothelial cell relaxation factor) production, and cell surface procoagulant activity. Transcription of mRNA and protein synthesis are required to produce these profound metabolic effects of both IL-1 and TNF-α, and thus the intracellular signal transduction pathways activated by these cytokines are potential targets for inhibition. Both IL-1 and TNFα activate major protein kinase systems such as protein kinase C (PKC) and protein kinase A (PKA). Additional cytosolic proteins are usually the targets of these kinases, and in the case of TNFα, a 26-Kd protein is rapidly and transiently phosphorylated exclusively on serine residues. Inside the nucleus, transcription factors such as nuclear factor (NF)-κB, AP-1, and interferon regulatory factor 1 are induced. There are at least two mechanisms by which TNFα and IL-1 can cause cell death. The first is by activation of phospholipase A2 (PLA2). Arachidonic acid metabolites are observed in supernatants of culture exposed to cytokine before cell death, and quinacrine, a PLA2 inhibitor, completely protects against cytokine toxicity. A second mechanism by which cytokines mediate cytotoxicity is the production of intracellular hydroxyl radicals. The sensitivity of several cell lines to cytokine toxicity appears to be inversely related to the radical scavenging capacity of those cells, and compounds that inhibit the production of free radicals can prolong cell survival when exposed to TNFα or IL-1. It is likely that the production of IL-1 and TNFα can also stimulate the release of cytokines from the IL-8 family, which in turn help to recruit and activate secondary cellular effectors (eg, neutrophils, NK cells, and monocytes) as well as contribute directly to capillary fragility, edema, and cell death. Other cytokines may also be part of this network; eg, macrophage-derived NKSF may stimulate NK cells to produce INFγ and to display enhanced cytolytic activity against host tissues. Recent data indicate that the frequency of lymphocytes producing IFNγ mRNA is dramatically increased in mice with acute GVHD. Such observations suggest that the effects of cytokines such as NKSF may be enhanced if the frequency of responding cells increases. Thus, multiple inflammatory cytokines may activate cytotoxic cells to cause direct damage to target organs, but it is probable that the role of cytokines as direct mediators of tissue destruction is more important than had been realized previously.

RELATIONSHIP OF ACUTE GVHD TO INFECTION

Several lines of evidence indicate that infection-induced cytokine production and GVHD are closely linked. Allogenic transplants in germ-free mice have a greatly reduced incidence of acute GVHD. When the transplanted animal is selectively contaminated with a single species of bacteria, organisms such as S epidermidis and lactobacillus cause no problems. However, the introduction of gram-negative rods causes fatal acute GVHD. A similar effect has been observed in humans. Patients with aplastic anemia transplanted in laminar air flow rooms with gut/skin decontamination have less acute GVHD and a better survival than patients treated in conventional rooms. The effect of a sterile environment is less apparent in patients with leukemia, possibly due to the use of more intensive conditioning regimens or to physiologic changes in the host associated with the disease itself. There is much anecdotal evidence that acute GVHD has a tendency to flare in association with clinical infections. In this context it is important to note that the growth of certain virulent Escherichia coli strains is enhanced by IL-1, suggesting that the local production of IL-1 in the gut mucosa can amplify any
putative effects of endotoxin or viable E coli. Finally, as noted above, controlled trials show that high-dose γ-globulin can reduce the risk of acute GVHD. This effect may be mediated in part by a reduction in gram-negative infections, but γ-globulin may also have the immunomodulatory effects discussed above.

One attractive aspect of the cytokine hypothesis is its ability to explain the selectivity of target organs in GVHD. Skin, gut, and liver are all barriers to microorganisms. Although the role of the liver in this regard is initially less obvious than the role of the skin and gut, the liver also functions as a barrier to endotoxin and gram-negative organisms that pass through the intestinal mucosa. The lung is also now thought to be a GVHD target organ. The bronchial epithelium is likewise an important barrier to infection, but its exposure to gram-negative organisms is much less intense than the other target organs, and therefore its position as a target organ is less well established. However, it is possible that this relationship partially accounts for the association between acute GVHD and interstitial pneumonia and perhaps hemorrhagic pneumonitis.

SUMMARY

We suggest that acute GVHD after marrow transplantation reflects (1) host injury due to the conditioning regimen followed by the production of inflammatory cytokines; (2) stimulation of mature donor T cells in the milieu of increased cell surface expression of leukocyte adhesion molecules and HLA molecules, followed by the autocrine production of IL-2; and, finally, (3) recruitment and activation of additional mononuclear effector cells from donor marrow progenitors, which produce additional inflammatory cytokines, thus sustaining the response. The second step is critical for the amplification of the systemic inflammatory response, and it is absent in autologous, syngeneic, and T-cell-depleted transplants. These T cells may also contribute to the inflammatory cytokine network. Acute GVHD can occur in the absence of primary tissue injury in such settings as transfusion-related GVHD; however, it is likely that a greater HLA disparity between donor and host is required. We propose that inflammatory cytokine production is the final common pathway of acute GVHD. If this model is correct, control of cytokine dysregulation at any of several points should control GVHD. Further studies of GVHD and investigations of cytokine antagonists (eg, IL-4 or IL-10) or combinations of antagonists such as IL-1ra and soluble TNF receptor or pentoxyfylline will allow us to determine the validity of this hypothesis.

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