Bone marrow transplantation (BMT) is often followed by significant morbidity and mortality due to protracted immunodeficiency. We have hypothesized that the bone marrow-ablative regimen may delay the recovery of normal immune function following transplantation by impairing the interaction of host endothelial cells with circulating graft-derived lymphocytes. This report compares the relative effects of busulfan (an alkylating drug) and gamma-irradiation on the tissue-specific localization potential of lymphocytes and the eventual recovery of immune function within syngeneic murine transplant recipients. Localization of normal lymphocytes into peripheral lymph nodes of irradiated BMT recipients was markedly less (~50%) than in busulfan-treated or normal mice over the first 2 months post-BMT. This finding correlated with irradiation-induced endothelial cell edema and microvascular occlusions within lymphocyte-receptive areas of the nodal microvasculature. The effect of both preparative regimens on the recovery of contact hypersensitivity (CHS) was also analyzed. This response recovered more quickly (between 1 and 2 months) in busulfan-pretreated animals. Further experiments demonstrated that the decrease in CHS responsiveness appeared, in part, related to a depression in the capacity of lymphocytes to localize into skin sites of antigen deposition within irradiated mice. The impairment of tissue-specific lymphocyte localization may represent a novel mechanism by which whole body irradiation can contribute to delayed lymphocyte localization following bone marrow transplantation.

Bone marrow transplantation (BMT) has gained widespread acceptance in the treatment of a wide range of clinical conditions, including congenital immunodeficiency, aplastic anemia, and leukemia. While representing a major advance in the treatment of these diseases, the procedure is also associated with significant morbidity associated with severe protracted alterations in immunologic function. Depressions of both humoral and cellular immune functions occur during the first 100 days after transplantation. This pan-immunodeficiency is thought to represent a consequence of the aggressive chemotherapy or radiotherapy protocols used to ablate the recipient’s immunohematologic system, before donor marrow infusion.

The eventual hematologic and immunologic recovery post-BMT is closely linked to the engraftment, proliferation, maturation and differentiation of donor-derived stem cells. Recovery of peripheral blood lymphocyte subsets can usually be demonstrated within 3 to 6 months of BMT. Despite the relatively early reappearance of phenotypic T and B cells, functional immunologic recovery may take over 2 years. During this period, serum antibody production, cytotoxic T cell function, mitogen responsiveness, as well as other lymphocyte functions appear to recover. Some complex immunologic responses, such as antigen-specific helper T cell function, IgA secretion, and contact hypersensitivity responses, appear to be even more delayed in their recovery.

The degree of histocompatibility between the bone marrow donor and recipient does not appear to significantly influence the rate at which immunologic recovery occurs following BMT. Immunologic reconstitution occurs at a similar pace following human twin, allogeneic and autologous transplants. The development of graft-v-host disease, however, significantly worsens the severity and duration of the various cellular and humoral immune deficits observed post-transplantation.

It is possible that current BMT protocols have detrimental effects on the recovery of the immune system post-transplantation by producing unintended effects on host tissues. We have hypothesized that some of the post-BMT immunologic deficits are produced by toxicity of the bone marrow-ablative regimen on the endothelial cells of the recipient. Alterations in the capacity of endothelial cells to bind graft-derived lymphocytes may account for decreased tissue localization potential of immune effector cells and thereby result in depressed immunologic function.

Under normal circumstances, mature lymphocytes continually migrate throughout the body, passing from the bloodstream through the various lymphoid organs and eventually returning to the bloodstream. The ability of lymphocytes to migrate through tissues is termed recirculation. Virtually the entire lymphocyte content of peripheral lymph nodes enters the nodal parenchyma from the bloodstream via this process. Tissue-specific extravasation appears to require the selective interaction of lymphocyte surface receptors with lymph node microvascular endothelial cells. The adhesion process appears to take place in histologically distinct cuboidal endothelium-lined post-capillary venules, known as high endothelial venules (HEV). Analogous tissue-specific extravasation-mediating structures are also associated with the endothelium of other lymphoid organs, such as the...
Peyer's patches,24 mesenteric lymph nodes,23 peribronchial lymphatic tissue,25 and synovium.26

Continual lymphocyte recirculation through peripheral lymph nodes is believed to enhance the probability that a T cell, which is committed during differentiation to a single antigen epitope, can encounter its specific antigen.23,27 The antigen recognition process is aided by the simultaneous concentration of antigen-presenting cells (APCs) within peripheral lymph nodes via the afferent lymphatic drainage.28 The peripheral lymph nodes also contain all of the humoral and cellular regulatory elements needed to amplify or depress immune responsiveness. The peripheral lymph nodes, therefore, serve as structures that facilitate the antigen recognition process and enhance immune surveillance of host tissues.

Our previous studies established that marrow-ablative doses of gamma-irradiation (>7.5 Gy) caused distinctive ultrastructural changes in murine and human lymph node HEV.29 These alterations in murine HEV were associated with a depressed capacity of lymphocytes to localize into irradiated lymph nodes from the bloodstream.29 Due to the importance of peripheral lymph nodes in the initiation and regulation of immune responses, we hypothesized that this depressed localization capacity should be associated with demonstrable changes in immune function.

In this report we compare the effects of two commonly used bone marrow-ablative agents,2 busulfan (an alkylating drug) and gamma-irradiation, on HEV histology and lymphocyte tissue localization potential. Since the two preparative regimens differed significantly in their endothelial toxicity, it proved possible to assess their respective effects on the timecourse of immunologic reconstitution following BMT.

MATERIALS AND METHODS

Animals. Six- to ten-week-old C3H/HeN mice were obtained from the animal production facility of the National Cancer Institute (Bethesda, MD). All mice were housed at a maximum density of six animals per 18 x 28 cm cage and maintained on Wayne sterile diet (Wayne Lab Blox, Wayne Pet Food, Chicago) and acidified water ad libitum. Mice were age and sex matched for each experiment. Before exposure to either preparative regimen, the experimental animals were pretreated for two days with 0.5 mg of gentamycin sulfate administered intraperitoneally (IP). Throughout the course of the experiment, recipient mice also received oral nonabsorbable antibiotics (35 mg of neomycin and 2,500 units of polymyxin B per 200 mL of drinking water). This treatment was used to reduce mortality due to infection during the experiment.

Preparation of lymphocytes. Peripheral lymph node (PLN) (inguinal, axillary, brachial, and cervical) were excised from donor mice and gently dissociated in RPMI 1640 medium (GIBCO, Grand Island, NY) with 5% fetal bovine serum (Sterile Systems, Logan, UT) (complete medium). The cell suspension was layered onto a glass-wool column to remove adherent as well as nonviable cells. Cell suspensions were always ≥99% viable by trypan blue exclusion.

Gamma-irradiation of mice. Recipient mice were lethally irradiated with 7.5 Gy (1 Gy = 100 rad) per whole body at 4.8 Gy/min using a Gamma-irradiator (Isomedix, Parsippany, NJ) containing a cesium-137 source. Animals were autopsied, and individual organs were removed and placed into phosphate-buffered saline (PBS, pH 7.4). Lymphocytes were isolated from antigen-sensitized donor mice, and a single cell suspension prepared (5 x 108 cells/mL). Each recipient was infused IV with 4 x 107 lymphocytes per milliliter in RPMI and 0.2 mL was injected intravenously (IV) into normal, busulfan-treated, or gamma-irradiated recipient mice. The administration of gamma-irradiation or busulfan was timed in such a manner that the comparative localization studies involving the infusion of 125I-labeled lymphocytes into the various experimental groups could all be performed from a single preparation of radiolabeled cells.

Radiolabeling of lymphocytes with 125I for localization studies. Cells synthesizing DNA following antigen sensitization were labeled with 125I (as iododeoxyuridine [3H1UDR], Amersham, Inc., Arlington Heights, IL). Lymphocyte suspensions (106 viable cells/mL) were incubated with 100 μCi [125I]Cr for half an hour at 37°C. Radiolabeled cells were washed three times in complete medium, adjusted to 107 lymphocytes per milliliter in RPMI and 0.2 mL was injected intravenously (IV) into normal, busulfan-treated, or gamma-irradiated recipient mice. The administration of gamma-irradiation or busulfan was timed in such a manner that the comparative localization studies involving the infusion of 125I-labeled lymphocytes into the various experimental groups could all be performed from a single preparation of radiolabeled cells.

Isotope quantitation and data presentation. Animals were killed four hours after infusion of labeled cells. The mice were autopsied, and individual organs were removed and placed into separate vials for counting in a Beckman Gamma 8000 gamma counter (Beckman Instruments, Irvine, CA), as was the remaining carcass. Data are presented as the percentage of radioactivity recovered per individual organ, as compared with the total radioactivity recovered from the experimental animal. Background values were subtracted from each one-minute count. This method of data calculation precludes errors introduced by inoculation variation between animals, as organ distribution is independent of the number of radiolabeled cells injected over a wide dose response range.32 Localization experiments were run using two to three animal groups because of high concordance between animals. Each experiment was repeated at least twice.

Assessment of relative marrow ablative potential of irradiation and busulfan regimens. Relative bone marrow stem cell ablation produced by gamma-irradiation (a single dose of 7.5 Gy) or busulfan (25 mg/kg/d for six days, IP) was quantified by an in vitro granulocyte-macrophage colony assay (CFU-GM). One femur from each mouse within three animal groups (which had previously been exposed to one of the preparative regimens) was isolated, and the marrow irrigated out with a syringe. Each femur contains ~4.5% of the total mouse bone marrow compartment.28 Following gentle dissociation of each animal's marrow into a single cell suspension, erythrocytes were lysed with Tris-buffered 0.16 mol/L ammonium chloride. The remaining nucleated cells were counted by phase microscopy. The CFU-GM were assessed by plating the entire nucleated bone marrow cell population from each femur into 2 mL of 0.8% methylcellulose (Dow Chemical Co, Midland, MI), in the presence of murine post-endotoxin serum (a source of GM-colony...
Sensitization and elicitation of contact hypersensitivity (CHS). Experimental and control mice were sensitized on the shaved abdomen with 25 μL of 0.25% 2,4-dinitrofluorobenzene (DNFB, Sigma, St. Louis) in a vehicle of acetone/olive oil (4:1) on two consecutive days. Four days after the first sensitization, a challenge dose of 10 μL of 0.25% DNFB was applied to the right hind footpad. Footpad swelling was measured (using a micrometer) 24 and 48 hours after challenge. The increment in footpad swelling of a normal control mouse was taken as the mean ± SEM. Immunoperoxidase staining was then performed using a modified avidin-biotin-peroxidase complex technique.36

Adoptive transfer of CHS. Normal mice were sensitized with 25 μL of 0.25% DNFB on the shaved abdomen and 10 μL DNFB on each footpad and ear on two consecutive days (donors). Four days after the initial sensitization, popliteal, inguinal, axillary, brachial, cervical nodes (including each ear separately) assayed to determine the localization of normal lymphocytes within BMT recipients. Mice were transplanted using murine bone marrow cells (106 cells/concentration). After 12 days colonies of >40 cells were counted. Normal mouse bone marrow cells (106 cells per 60 mm plate), cultured in the presence of GM-CSF served to establish the maximum colony formation potential. Bone marrow cells plated in the absence of GM-CSF served as a negative control.

Immunoperoxidase staining of frozen sections. Frozen sections were incubated for 30 minutes with MECA-325 (generously supplied by E. Butcher, Stanford, CA). MECA-325 is a monoclonal rat-anti-mouse endothelial cell antibody (IgGl), which was produced by fusing spleen cells from rats that had been immunized with mouse (Balb/c) lymph node stroma with SP 2/0 mouse myeloma cells.37 This monoclonal antibody binds specifically to murine HEV, and not other lymph node structures.38 No cross reaction occurs with capillaries, arteries, and arterioles that do not have the cuboidal endothelial features associated with specific lymphocyte binding.39 To identify MECA-325 binding, a biotinylated rabbit-anti-rat antiserum (Cappel) was used as a second stage reagent. This reagent did not bind to mouse lymph node sections in parallel control slides. Immunoperoxidase staining was then performed using a modified avidin-biotin-peroxidase complex technique.36

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In some experiments, the antigen-sensitized lymphocytes were radioiodolated with [125I]UDR before their adoptive transfer. In these experiments, the animals were challenged on one ear (instead of the footpad), and the increment in ear swelling (× 10-4 inches) was measured only at 24 hours. Each animal was then killed and its organs (including each ear separately) assayed to determine the distribution of the infused radioiodolated cells (as described above).

Direct injection of hapten-modified lymphoid cells and effector lymphocytes into the skin. Syngeneic spleen cells were treated with buffered ammonium chloride to lyse erythrocytes (as described above). Splenocytes were trinitrophenylated by incubation of suspensions of 106 cells/mL in PBS containing 2.5 mmol/L 2,4,6-trinitrobenzene sulfonic acid (TNBSO4) for 10 minutes at 25°C. After hapten modification, the cells were extensively washed in medium and resuspended at 106 cells/mL to use as targets for antigen-primed effector cells. The effector cells were generated by sensitizing mice on the belly, footpad, and ears (as described above) with a single application of 7% 2-chloro-1,3,5-trinitrobenzene (TNCB). These mice subsequently served as donors for sensitized lymphocytes four days later. The antigen-sensitized lymphocytes were also suspended at 106 cells/mL, and mixed 1:1 with hapten-modified target cells. Groups of irradiated BMT recipients or normal control mice were injected with 10 μL of the combined cell suspension in the right ear. The increment in ear swelling (compared with the contralateral ear) was determined 48 hours later to minimize any residual swelling due to injection trauma (× 10-4 inches). Mice that received an equivalent volume of sensitized effector lymphocytes without hapten-modified splenocytes served as a negative control.

RESULTS
The initial experiment was designed to compare the relative effects of irradiation or busulfan exposure on tissue localization of normal lymphocytes within BMT recipients. These experiments were performed by infusing 2 x 106 normal [51Cr]-labeled peripheral lymph node lymphocytes IV into previously transplanted mice (0.2 mL/recipient). Four hours following the infusion of radiolabeled lymphocytes, the animals were killed and autopsied. Each organ (peripheral lymph nodes, spleen, mesenteric nodes, gut, liver, kidneys, lungs and the residual carcass) was counted separately in a gamma counter to determine the primary localization of the cells. The results (Fig 1) are expressed as a percentage of the injected cells that localized within the peripheral lymph nodes (±SEM), compared with the total recovery from each animal. As early as 1 week post-BMT, a time when the function of both the hematopoietic and immunologic systems of the BMT recipient remain ablated by the preparative regimen, lymphocyte localization into the lymph nodes of previously irradiated mice was <50% of controls. No evidence of lymphocyte sequestration in any other organ could be demonstrated as a cause for the depression in cells reaching the peripheral lymph nodes (data not shown).
not shown). The decrease in lymphocyte localization into peripheral lymph nodes in irradiated mice persisted through the 1- and 2-month timepoints. These times were chosen for experimental evaluation because significant graft-derived hematologic recovery occurs by 1 month post-BMT. By 2 months following syngeneic murine transplantation, phenotypic recovery of graft-derived lymphocytes in the blood and lymphoid tissues may be observed.

Busulfan-treated mice did not exhibit significant depression in the capacity of lymphocytes to localize into nodes at any time tested. This suggests that busulfan, unlike gamma-irradiation, does not adversely affect the lymphocyte-receptive endothelium within peripheral lymph nodes.

These results could also be explained if the marrow-ablative dose of busulfan was less potent than that of gamma-irradiation. If this were true, less lymph node microvascular damage might also be expected. We performed an experiment to directly assess the degree of bone marrow stem cell ablation produced by both of the experimental regimens. Groups of three mice were pretreated with either busulfan or irradiation. Hematologic reconstitution by BMT was not performed. Twenty-four hours after the last exposure to either preparative agent, the marrow content of one femur per animal (~4.5% of its total bone marrow) was assessed for its ability to form CFU-GM. Care was taken to assess CFU-GM in busulfan-pretreated mice at a time when all drug and its active metabolites were believed to have been cleared from the circulation.

To establish the normal CFU-GM content per femur, a group of normal age- and sex-matched control mice served as a control. The results of this experiment (Table 1) established that busulfan-pretreatment decreased the number of CFU-GM by a factor of >10^3/femur, compared with 10^4 for 7.5 Gy-irradiated animals. The apparent differences in lymphocyte recirculation potential produced by the two regimens cannot, therefore, be accounted for by less intensive marrow ablation within the busulfan-treated group. These two regimens likely differ in their effects on the lymph node HEV structures.

The microanatomy of the peripheral lymph node HEV was directly examined by immunohistochemical techniques. Frozen sections of mouse peripheral lymph nodes were prepared from animals 2 months postexposure to either busulfan or irradiation. These sections were stained with the monoclonal antibody, MECA-325, using an immunoperoxidase technique. This monoclonal antibody specifically identifies lymphocyte-receptive areas of endothelium within peripheral lymph nodes (high endothelial venules). The immunoperoxidase-stained sections of gamma-irradiated lymph nodes (Fig 2A) demonstrated a marked disorganization of the HEV histology, with apparent edema and proliferation of the endothelial cells, producing microvascular occlusions. Lymph nodes from busulfan-pretreated animals (Fig 2B) were found to have a normal appearing HEV architecture, with cuboidal endothelium and patent vascular lumens. A MECA-325-stained section of a lymph node from a normal animal is presented for comparison (Fig 2C). Electron microscopy (EM) was also performed at the 2-month timepoint. The EM studies confirmed our previous findings of severe intracellular edema within endothelial cells of previously irradiated HEV. Lymph node endothelial cells from busulfan-pretreated mice, on the other hand, were ultrastructurally normal (data not shown).

Since the capacity of lymphocytes to enter peripheral lymph nodes appears to be impaired by prior irradiation, it follows that immunologic hyporesponsiveness might occur as a consequence. Contact hypersensitivity (CHS) was the initial response evaluated, because CHS is known to be depressed for years following BMT in humans. CHS may be particularly susceptible to preparative-regimen-induced endothelial damage, because lymphocyte recirculation is thought to play an important role in this response. The sensitization and amplification phases of this response take place, in part, following localization of circulating lymphocytes into the region lymph nodes that drain the antigen-exposed skin. Antigen-sensitized CHS-effector T cells that are subsequently generated in the peripheral lymph nodes are thought to exit the node via efferent lymphatics and migrate via the thoracic duct into the bloodstream. These sensitized lymphocytes exhibit the capacity to extravasate from the bloodstream specifically into sites of subsequent antigen exposure in the skin.

The effects of the BMT-preparative regimen on the induction of CHS-responsiveness were assessed by testing groups of mice transplanted using either busulfan or irradiation 1 and 2 months previously. Mice were sensitized with DNB on their shaved abdominal skin. Four days after the initial sensitization, the challenge dose of DNB was applied to the right hind-footpad. The degree of swelling was measured with a micrometer 24 hours later, and compared with the contralateral normal footpad. A group of sensitized and challenged age-matched normal mice served to establish the normal range of CHS-responsiveness for each experimental timepoint. A nonsensitized group that was exposed only to

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Table 1. Cytoreductive Potential of the Irradiation

and Busulfan Treatment Protocols

<table>
<thead>
<tr>
<th>Group</th>
<th>Nucleated Cells Recovered per Femur</th>
<th>CFU-GM Recovered per Femur</th>
<th>Stem Cells Reduction by Preparative Regimen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.6 x 10^7</td>
<td>11,200</td>
<td>0</td>
</tr>
<tr>
<td>Irradiation</td>
<td>5.0 x 10^8</td>
<td>7.8</td>
<td>99.03</td>
</tr>
<tr>
<td>Busulfan</td>
<td>3.4 x 10^8</td>
<td>0.8</td>
<td>99.003</td>
</tr>
</tbody>
</table>

The decrement in bone marrow stem cells within animals exposed to either busulfan (25 mg/kg/d × 6 days IP) or irradiation (7.5 Gy) was quantified with the CFU-GM assay. Three animal groups were treated with each of the preparative regimens. Twenty-four hours following completion of the marrow-ablative regimen, a single cell suspension was prepared from the bone marrow contained within one femur from each animal. Erythrocytes were lysed with buffered ammonium chloride and the number of nucleated cells counted. The entire nucleated cell content of each femur (~4.5% of the total marrow mass of a mouse) was then plated into 0.8% methyl cellulose in RPMI 1640 medium, 20% fetal bovine serum, and a predetermined optimal concentration of murine post-endotoxin serum (a source of GM-CSF). After 12 days, the resulting CFU-GM colonies (~40 cells) were counted. The CFU-GM content of normal femurs was established by plating 10^6 nucleated bone marrow cells, derived from age-matched controls, into culture as described above.

The CFU-GM content of a normal femur was then calculated based on:

(10^6 cells) x (CFU-GM/10^6 cells plated into culture).

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...completed with the CFU-GM assay. Three animal groups were treated with each of the preparative regimens. Twenty-four hours following completion of the marrow-ablative regimen, a single cell suspension was prepared from the bone marrow contained within one femur from each animal. Erythrocytes were lysed with buffered ammonium chloride and the number of nucleated cells counted. The entire nucleated cell content of each femur (~4.5% of the total marrow mass of a mouse) was then plated into 0.8% methyl cellulose in RPMI 1640 medium, 20% fetal bovine serum, and a predetermined optimal concentration of murine post-endotoxin serum (a source of GM-CSF). After 12 days, the resulting CFU-GM colonies (~40 cells) were counted. The CFU-GM content of normal femurs was established by plating 10^6 nucleated bone marrow cells, derived from age-matched controls, into culture as described above. The CFU-GM content of a normal femur was then calculated based on:

(10^6 cells) x (CFU-GM/10^6 cells plated into culture).
Fig 2. Immunoperoxidase staining of peripheral lymph node HEV with the monoclonal antibody MECA-325. Mice were exposed to either irradiation (A) or busulfan (B) marrow ablation, followed by syngeneic hematopoietic reconstitution. Frozen sections (6 μm) were prepared from their lymph nodes, obtained 2 months after BMT, and stained with MECA-325 by immunoperoxidase technique as described in the Materials and Methods section. The irradiated lymph nodes exhibit apparent endothelial cell proliferation, edema, and occlusion of vascular lumens, unlike busulfan exposed nodes. For comparison, a normal peripheral lymph node is also shown (C).
the challenge dose of DNFB served as a negative control. This experiment (Fig 3) demonstrated that the de novo induction of CHS-responsiveness was markedly diminished in both the irradiated and busulfan-treated groups at 1 month posttransplantation. By 2 months, however, the busulfan group had recovered a normal degree of CHS-responsiveness. The 2-month irradiated group, on the other hand, still exhibited a markedly depressed response.

These results indicate that this antigen-specific immunologic response is depressed longer in previously irradiated mice than in busulfan-pretreated BMT recipients. The elicitation of CHS responses, however, requires a complex system that involves multistep antigen sensitization, amplification, and effector phases. Any experimental result must therefore be interpreted with caution, since irradiation could be acting at one or multiple steps in the response. Additional experiments were performed, analyzing two specific components of the CHS response, to better define some of the potential mechanisms resulting in the observed depression of CHS responsiveness.

The effector phase of the CHS response (ie, the ability of sensitized effector lymphocytes to localize into the skin from the bloodstream and produce edema) can be tested by means of an adoptive transfer experiment. Such an experiment was performed by allowing antigen sensitization to proceed in normal animals. A single-cell suspension of lymphocytes was prepared from the lymph nodes of these donor mice, and $4 \times 10^7$ cells/mouse were infused IV into groups of experimental animals. This experiment was performed in groups of mice transplanted 1 and 2 months previously, using either the busulfan or irradiation protocol. Mice were challenged with antigen on one footpad immediately following the adoptive transfer of cells. The increment in footpad swelling was determined 24 hours later (Fig 4). Parallel groups of normal age-matched control mice were used at each time point to establish the maximum expected degree of swelling elicited by such an adoptive transfer of effector cells. The results of this experiment (Fig 4) demonstrate a marked reduction in the capacity of normal DNFB-sensitized effector lymphocytes to elicit swelling in footpad challenge sites in previously irradiated mice (28% and 34% of control at 1 and 2 months post-BMT, respectively). This experiment suggests that prior radiotherapy interferes with either the ability of effector T cells to localize into the skin of irradiated BMT recipients or the subsequent ability of these cells to elicit swelling within cutaneous tissues. The response was somewhat depressed at 1 month following busulfan exposure. By 2 months, however, the ability to adoptively transfer CHS responsiveness into busulfan-treated recipients was not different from normal. Measurement of footpad swelling at 48 hours (to exclude the possibility that irradiated animals exhibited a delayed, but otherwise normal, CHS response) demonstrated a similar pattern to the 24-hour measurements, but were uniformly reduced in magnitude within each group of mice.

The possibility that previous irradiation of the skin is responsible for the failure to produce edema was then addressed. Four days after sensitization of normal donor

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**Fig 3.** The induction of CHS in BMT recipients. Groups of mice (4 to 5 animals/group) were transplanted with busulfan or gamma-irradiation 1 or 2 months before the onset of the experiment. These animals were sensitized to 0.5% DNFB on two consecutive days, as described in the Materials and Methods section. Four days later, the animals were challenged on the right hind footpad with 10$\mu$L 0.5% DNFB. The increment in swelling of the right hind-footpad (compared with the contralateral unchallenged footpad) was measured by micrometer 24 hours later, and is recorded as units ($10^{-4}$ inches) ± SEM. Normal mice that were sensitized and challenged or nonsensitized animals that received only the challenge dose of DNFB served as positive and negative controls, respectively.

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**Fig 4.** The adoptive transfer of CHS to previously transplanted mice. Normal mice were sensitized to DNFB and served as donors for the adoptive transfer of antigen-sensitized peripheral lymph node lymphocytes. A single cell suspension of peripheral lymph node lymphocytes from these mice ($4 \times 10^7$ cells per recipient) was injected IV into groups of previously transplanted mice (1 or 2 months post-BMT). These recipients were immediately challenged on the right hind-footpad with 10$\mu$L 0.5% DNFB. Twenty-four hours following challenge, the increment in footpad swelling was measured ($10^{-4}$ inches ± SEM).
mice with trinitrochlorobenzene (TNCB), donor animals were killed and their lymph nodes excised. A single cell suspension was prepared from the sensitized lymphocytes contained within these nodes. An equal number of these cells and antigen-haptenized spleen cell targets were mixed in a small volume of media, and 10 µL injected into the right ear pinnae of mice that had been irradiated 2 or 8 months previously. The increment in ear swelling was measured with a micrometer 48 hours after injection, to minimize any degree of edema attributable to injection trauma. A parallel group of normal mice injected with sensitized effector lymphocytes and antigen served as a positive control. Animals that received only the same number of antigen-primed lymphocytes without antigen (in equivalent volume of medium) served as a negative control. The results of this experiment (Fig 5) demonstrated that the degree of ear swelling that could be elicited in both the irradiated and unirradiated groups was similar. The response was also shown to be antigen dependent, since animals that received only sensitized lymphocytes, but no antigen, did not develop ear swelling. The conclusion of these experiments is that irradiated skin is capable of responding to a local CHS stimulus with the same degree of edema as normal skin.

These experiments suggested that irradiation-induced depressions in CHS responsiveness were, in part, due to a decrease in the ability of effector lymphocytes to localize into the skin from the bloodstream. This possibility was experimentally tested by preparing a single cell suspension of DNF-sensitized lymphocytes derived from normal mice. Proliferating cells within this suspension (presumably undergoing clonal expansion in response to antigen exposure) were labeled with 125IUDR for two hours. Cells from this suspension (4 x 10^7 cells/recipient) were then infused IV into previously transplanted mice. These animals were immediately challenged with DNF on the right ear. As in preceding adoptive transfer experiments, the increment in swelling was measured at 24 hours. The mice were subsequently killed and autopsied. Each ear (as well as other organs and carcass) were counted separately in a gamma counter to determine the percentage of the recovered radiolabel within the antigen-challenged ear site compared with the non-challenged ear. The results of this experiment (Table 2) confirmed the previously noted depression in the capacity to adoptively transfer CHS to previously irradiated recipients. The depression in edema generation was paralleled by a depression in the influx of radiolabeled lymphocytes into this site. Busulfan treated mice exhibited a depression in lymphocyte localization into the ear challenge site (and depressed CHS responsiveness) 1 month post-BMT. By 2 months, however, the ability of adoptively transferred effector lymphocytes to elicit edema and enter sites of antigen challenge was significantly better than their irradiated counterparts, and approached the response within normal age-matched (control) recipients.

**DISCUSSION**

The optimal preparative regimen for use in human BMT is a controversial issue, centered mainly around the narrow...
ablation to relatively low doses of gamma-irradiation (>7.5 Gy) has been shown to produce increased vascular permeability, vasodilation, and intimal swelling, particularly within the skin. These changes are believed to be reversible. Radiation-induced vascular fibrosis and decreases in capillary density have not generally been observed with doses <20 to 40 Gy. Our previous studies in mice established that exposure of the lymph node microvasculature to relatively low doses of gamma-irradiation (>7.5 Gy) caused significant anatomic changes. Endothelial cell edema and microvascular occlusions were observed within areas of lymphocyte-receptive endothelium (HEV), which persisted for more than 6 to 8 months post-BMT. These histologic findings correlated with a depressed capacity for normal lymphocytes to enter irradiated lymph nodes from the bloodstream. Similar changes were found on examination of lymph nodes derived from irradiated human BMT recipients up to 1 year post-BMT. The lesions in both murine and human BMT-recipients were histologically distinct from previously described radiation-induced microvascular lesions.

The current investigation established that busulfan had little effect on lymphocyte recirculation and HEV anatomy in mice. While irradiation caused apparent endothelial cell proliferation, intracellular edema, and microvascular occlusions, busulfan did not affect the appearance of HEV structures. These anatomic changes correlated with a depression in the ability of lymphocytes to localize into the lymph nodes of irradiated, but not busulfan-treated mice.

Since lymph nodes are believed to be important sites for antigen recognition and amplification of immune responses, we hypothesized that changes in lymphocyte localization into these organs following BMT would be accompanied by demonstrable depressions in immune function. We compared the effects of busulfan and irradiation on the reconstitution of CHS responsiveness in transplant recipients. CHS is believed to be an anatomically restricted (compartmentalized) immune response that involves a circuit that Streilein has termed the skin-associated lymphoid tissue (SALT). Lymphocyte tissue localization is believed to be necessary for this response. During the afferent limb (ie, antigen sensitization), the continual migration of T cells into peripheral nodes contributes to foreign antigen recognition and the development and clonal expansion of CHS-effector cell populations. The antigen-sensitized effector lymphocytes are also believed to gain the capacity to localize selectively from the bloodstream to sites of antigen challenge in the skin.

Our studies demonstrated that CHS responsiveness recovered more quickly in busulfan-treated mice than in irradiated animals. The cause of the depression of CHS responsiveness in irradiated animals is difficult to interpret, since one step or multiple steps in the antigen sensitization and effector phases of the response could be affected by irradiation. Subsequent experiments established that one of the mechanisms that may contribute to a diminished CHS response in irradiated animals was the depressed capacity of lymphocytes derived from normal donors to localize into irradiated skin sites. Since proliferating cells were labeled in these studies, our results may reflect an irradiation-induced depression in the skin localization of the CHS effector cells. It should be remembered, however, that recruited, non-antigen-sensitized lymphocytes contained in the adoptively transferred population may also contribute to the cellular influx into the skin. The role of each cell population in the generation of the CHS response is as yet not fully characterized. Our preliminary studies appear to indicate that the ability of the recruited (non-antigen-sensitized) lymphocyte population to enter the skin is also significantly depressed following irradiation (unpublished data). Locally injected effector cells, on the other hand, appear to have a normal capacity to elicit skin edema, and are unaffected by the prior irradiation. This appears to exclude the possibility that irradiation alters the ability of the skin itself to respond to cellular and soluble stimuli required to elicit the CHS response.

The capacity of normal CHS effector cells to migrate into the skin of busulfan-treated recipients was also depressed at early times post-BMT, perhaps due to a transient effect of the drug on lymphocyte receptive endothelium. Two months after BMT, the capacity of CHS effector cells to enter the skin of busulfan-treated mice had recovered, unlike their irradiated counterparts. The two preparative regimens, therefore, appear to exhibit differing effects on the interaction of circulating normal lymphocytes with the skin microvasculature. Whether this difference is due to alterations in lymphocyte binding to endothelial cells or anatomic occlusions of vessels (as in irradiated peripheral lymph nodes) remains to be established. These experiments have, for the first time, correlated the lymphocyte tissue localization process and the restoration of normal immune function post-BMT.

There have been a number of human BMT studies that have attempted to define the recovery of contact hypersensitivity following transplantation. This immune response appears to be depressed for years following human BMT. The majority of these studies were based on limited numbers of patients. These reports also do not distinguish between de novo induction of contact hypersensitivity and cellular "memory" responses to recall antigens.

Witherspoon et al recently reported the results of skin testing in 332 allogeneic and twin transplants. These investigators found that skin test responses to recall antigens remained depressed for up to 4 years following transplantation. The de novo induction of CHS to DNB was also assessed. This response appeared to recover 2 years or more after transplantation. No differences could be detected in the percentage of responding patients or in the timecourse of
CHS recovery between twin and allogeneic transplant recipients. The presence of acute or chronic graft-v-host disease, however, was found to significantly depress the frequency of positive skin test responses. Patients prepared with total body irradiation alone were hyporesponsive to DNFB sensitization for a more protracted period following transplantation than were patients treated with cyclophosphamide alone (an alkylating agent).3 Patients pretreated with a combination of the two agents (presumably with attenuated doses of both) responded more like the cyclophosphamide pretreated group.3 The findings of Witherspoon et al appear to support our conclusion that alkylating agents may be less detrimental to the recovery of CHS responses than total body irradiation, but caution must be used in extrapolating between the two studies. Human BMT patients represent a more complex system than our syngeneic murine transplantation model, and Witherspoon's patients were treated with a different set of preparative regimens than we used (with potentially differing toxicities). Despite these differences, similar mechanisms may contribute to the irradiation-induced depression in CHS responsiveness observed within both studies.

Other mechanisms are also likely to contribute to the depressed CHS responses seen in transplant recipients. Delayed T cell precursor proliferation and maturation in the posttransplant period may depress cellular immune function.3,4,5,9 Depressions in the function of irradiated thymic epithelium may contribute to the delayed reconstitution of functional T cell subsets in secondary lymphoid organs.9 Reconstitution of the T cell repertoire may not necessarily be complete following BMT. Relative imbalances in the number and functional capacities of helper and suppressor T cells may also develop and result in dominant suppression of immune responses.9,10

It is attractive to speculate that irradiation-induced depression of lymphocyte migration into the peripheral lymph nodes also contributes to the depressed recognition of antigens applied to the skin (ie, the afferent phase of the immune response). It is difficult, however, to establish this point experimentally. A number of distinct intracellular, intercellular, and cytokine-mediated processes are required for effective antigen sensitization and clonal expansion of effector lymphocyte populations. Dissecting the various elements of the afferent phase from each other in vivo is not a simple task. Experiments designed to estimate the relative efficiency of CHS effector cell generation within the peripheral nodes of irradiated v busulfan-treated mice are now in progress. If the number of CHS-effector cells within the nodes of irradiated animals is depressed, further studies will be necessary to establish the responsible mechanism(s).

We have intentionally used a simplified syngeneic transplantation protocol to avoid any potential interference with immunologic reconstitution by graft-v-host disease. The analysis of the marrow-ablative and immunologic consequences of single agents has allowed the comparison of their relative effects on microvascular anatomy, lymphocyte localization potential, and eventual immune reconstitution. This experimental model could easily be adapted for the evaluation of more complex preparative regimens. Such studies appear to be necessary to more accurately duplicate human BMT protocols. Most preparative regimens used in human BMT involve multiple, potentially interacting agents, to achieve host bone marrow ablation. Furthermore, immunomodulatory drugs such as methotrexate, azathioprine, cyclosporine A, and corticosteroids are often administered to allogeneic BMT recipients to decrease the frequency of graft-v-host disease. Each of these agents has the potential to interfere with lymphocyte localization into tissue. We have already demonstrated that corticosteroids act on endothelial cells and reversibly impair the ability of normal lymphocytes to enter peripheral lymph nodes and the skin following their parenteral administration to mice. Corticosteroids additionally appear to block CHS-effector cell functions following in vitro treatment of lymphocytes.50 Further investigation of the effects of BMT-preparative regimens on the immunologic reconstitution of the host may lead to effective marrow ablative regimens that minimize alterations in the lymphocyte recirculation process and allow rapid recovery of the immune system following transplantation.

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Recovery of contact hypersensitivity responses following murine bone marrow transplantation: comparison of gamma-irradiation and busulfan as preparative marrow-ablative agents

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