REQUIREMENTS FOR THE INDUCTION AND ADOPTIVE TRANSFER OF CYCLOSPORINE-INDUCED SYNGENEIC GRAFT-VERSUS-HOST DISEASE

BY ANNE C. FISCHER, WILLIAM E. BESCHORNER, AND ALLAN D. HESS

From The Bone Marrow Transplantation Program, Oncology Center, and the Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Cyclosporine (CsA), a potent immunosuppressive agent, is important in the treatment of several autoimmune diseases (1, 2) and the enhancement of graft survival (3–5), possibly facilitating the induction of transplantation tolerance. CsA in vitro inhibits the proliferative response of a primary MLR and prevents the development of cytotoxic T cells by interfering with the production of IL-2 and at higher doses the upregulation of its receptor (6). Its immunobiological role in tolerance induction, however, remains enigmatic. Although known as a potent suppressor of acute allogeneic graft-versus-host disease (GVHD) (7), CsA elicits an extensive autoimmune syndrome 14–21 d after the cessation of therapy after syngeneic bone marrow transplantation (8). This autoaggression syndrome is histologically indistinguishable from allogeneic GVHD. The occurrence of CsA-induced autoimmunity in rats autologously reconstituted from a shielded tibia reversed a landmark concept: no longer is histocompatibility disparity a requirement for GVHD, as once postulated by Billingham (9).

This autoimmune disease is mediated by T cells (10, 11) and is intimately associated with the insurgence of OX8+ (CD8) autocytotoxic T lymphocytes, which appear to recognize a public epitope on class II major histocompatibility antigens (12). A W3/25+ (CD4) helper subset appears also to play an important role in this disease (10). Previous studies have suggested that the requirements for the successful induction of syngeneic GVHD (SGVHD) include thymic irradiation and CsA treatment (8, 10). In addition, evidence has been presented that suggests that a host resistance mechanism must be eliminated before the development of this autoaggression syndrome (11).

In the present study we compare the specific requirements for the induction and adoptive transfer of SGVHD, which are two experimental modalities reflecting underlying processes fundamental to this complex autoimmune phenomenon. We reveal evidence that suggests that induction of SGVHD and host resistance to the adoptive transfer are two distinct mechanisms with different drug and radiation dose sensi-

1 Abbreviations used in this paper: CsA, cyclosporine; CY, cyclophosphamide; GVHD, graft-vs.-host disease; SGVHD, syngeneic GVHD.
tivities. Host resistance to adoptive transfer is mediated by an autoregulatory population that is thymic dependent, and sensitive to radiation and cyclophosphamide (CY).

Materials and Methods

Rats
Lewis (L,RT1) female rats, Cora virus free, and 6–8 wks old, were purchased from Charles River Breeding Laboratories (Wilmington, MA) and kept in isolation.

Radiation
Total Body Irradiation (TBI). Lewis rats were irradiated on day 0 with either 1050, 750, 500, or 300 rad at 108 rad/min from a dual-source 137Cs small animal irradiator (Atomic Energy of Canada Ltd., Kanata, Ontario, Canada).

Hemibody Irradiation. Lewis rats were irradiated in a 137Cs irradiator (J. L. Shepard Mark I, Glendale, CA) with a collimated beam. The attenuated dose directed either at the thymus or the pelvis was estimated to be 1,050 rad at 1 cm from the collimated beam without taking into effect the radiation dose absorbed in that 1 cm of intervening tissue. On day 0 of transplantation the rats received 50 × 10^6 splenocytes from autoimmune donors.

Other Preparative Regimens
CY. Lewis rats were injected intraperitoneally with 200, 100, 50, or 25 mg/kg of CY (Mead-Johnson, Evansville, IN) on day -1 before either the administration of a 30-d regimen of CsA therapy or the adoptive transfer of effectorsplenocytes.

Busulfan. Lewis rats were gavaged with 30 mg/kg of Busulfan (Burroughs Wellcome) on day 2 prior to the initiation of SGVHD or the adoptive transfer of effectorsplenocytes.

CY and Busulfan. Lewis rats were gavaged with 30 mg/kg of Busulfan (Burroughs Wellcome Co., Greenville, NY) on day -2 and injected intraperitoneally with 200 mg/kg of CY on day -1 before the initiation on day 0 of bone marrow reconstitution and CsA therapy for the induction phase.

Bone Marrow Transplantation
Donor viral-free Lewis rats were killed by CO2 asphyxiation. Marrow was collected from the femurs, tibia, and humeri in RPMI 1640. The marrow cells were adjusted to a concentration of 6 × 10^7 nucleated cells/ml and were infused into recipient animals by intravenous injection into the dorsal tail vein on day 0. The total volume injected was 1 ml.

Induction and Adoptive Transfer. To study the requirements for the induction of SGVHD, rats were treated with immunosuppressive therapy, infused with bone marrow, and subcutaneously injected with CsA for 30 d. To analyze the requirements for the transfer of disease, secondary recipients were either irradiated or treated with immunosuppressive therapy, and bone marrow was reconstituted and infused with effectorsplenocytes from autoimmune donors on day 0 unless otherwise indicated. Effectorsplenocytes were harvested from animals with early onset of SGVHD 10–14 d after CsA therapy after lethal irradiation and syngeneic marrow transplantation.

Antibiotics
Rats received medicated drinking water supplemented with bactrim, neomycin, and polymixin B as previously described (8).

Cyclosporine
CsA was the generous gift of Sandoz, Ltd., Basel, Switzerland. The powdered CsA was dissolved in 95% ethanol and added to a 5% emulphor solution in deionized H2O. Rats were weighed daily and received 1 ml/100 g/day subcutaneously from the day of marrow infusion for 30 consecutive days. The total dose of CsA per day per rat was 15 mg/kg. Control animals received the identical quantities of the drug diluent (ethanol, 5% emulphor, H2O) without CsA.
Assessment of GVHD

Rats were examined daily for signs of clinical GVHD such as red ears, alopecia, dermatitis, or diarrhea. Ear and skin biopsies were taken at frequent intervals. Previously described criteria were used for the histological documentation of acute and chronic GVHD (13, 14).

Monoclonal Antibodies

Murine mAbs directed against rat lymphocyte determinants were purchased from Serotec (Bioproducts for Science, Inc., Indianapolis, IN). The specific mAb from ascites fluid used in our studies consisted of W3/13 (pan-specific for rat T lymphocytes) and OX19 (pan-specific for rat T lymphocytes).

Cell Separation

Spleen cells from normal animals were fractionated over nylon wool (Fenwall Laboratories, Deerfield, IL) to enrich for T cells as previously described (15). The nonadherent population was >85% OX19+ by FACS analysis.

Panning. Nylon wool-nonadherent spleen cells from normal animals were depleted of T lymphocytes by panning, as previously described (16). Briefly, 10^7 lymphocytes, incubated for 1 h at 4°C with 0.2 ml of a 1:10 dilution of OX19 in PBS, were placed in petri dishes coated with affinity-purified goat anti-mouse IgG (Tago Inc., Burlingame, CA), the plates were lightly centrifuged and incubated for 1 h at 4°C. The nonadherent fraction was aspirated and reincubated with newly coated petri dishes for a second panning. The nonadherent fraction was again aspirated and the plates were carefully rinsed with a 0.2% BSA-PBS solution. The OX19-depleted population was analyzed with a directly FITC-conjugated W3/13.

Immunomagnetic Separation. Nylon wool-nonadherent spleen cells from normal animals were separated into lymphocyte subsets by immunomagnetic separation using immunomagnetic beads coated commercially with covalently bound, affinity purified (Fc specific) sheep polyclonal immunoglobulin against mouse IgG1 subclass (Dynabeads, Dynal Inc., Great Neck, NY) as previously described (17). Briefly, 5 x 10^7 cells were incubated with saturating concentrations of primary antibody for 1 h at 4°C on a hematology mixer (Fisher Scientific Co., Pittsburgh, PA). At the end of the incubation the supernatant was removed and the cells were thoroughly washed with PBS and 0.5% BSA three times. The cells were then incubated with the microspheres (one cell/five beads) for 30 min at 4°C on a hematology mixer. To remove the unbound cells from the suspension, the tube was held near a Dynal Magnetic Particle Concentrator for a few minutes, the suspension was removed with a Pasteur pipette, and the positively selected cells, complexed to the microspheres, were immobilized by the magnetic field.

FACS Analysis. After each type of cell separation listed above, the depleted populations were stained with an appropriate mAb and the single label immunofluorescence was analyzed on a Hewlett-Packard Co. (Palo Alto, CA) FACSCAN analytical scanner. The negatively selected populations depleted with OX19 either by panning or immunomagnetic separation resulted in <15% W3/13+ by immunofluorescence.

Results

The Role of Irradiation in the Induction and Adoptive Transfer of SGVHD. The current studies were designed to identify the specific levels of irradiation necessary for the manifestation of SGVHD by direct primary induction or by adoptive transfer.

For the induction studies, Lewis rats were subjected to graded doses of whole body irradiation followed by infusion of syngeneic marrow (6 x 10^7 nucleated cells) and treatment with CsA for 30 d. The incidence of SGVHD was assessed for each dose of radiation. Lethal total body irradiation of 1,050 rad on the day before bone marrow reconstitution and the start of CsA therapy correlated with a 100% incidence of clinical SGVHD as indicated in Fig. 1. At a sublethal dose of 750 rad, one-third of the rats tested demonstrated a severe clinical and histologic SGVHD. Lower doses of irradi-
atation tested were ineffective in allowing the induction of SGVHD. At all levels of
radiation control animals treated with mock diluent post marrow reconstitution
demonstrated no evidence of disease as did animals treated with CsA that did not
receive any irradiation.

For the adoptive transfer studies, Lewis rats were prepared with graded doses of
radiation followed by infusion of splenocytes from animals with active SGVHD to-
gether with normal marrow. Both lethal and sublethal doses of radiation elicited
a high incidence of severe SGVHD upon the infusion of effector splenocytes (Fig.
1). At 500 rad, one of seven rats demonstrated clinical SGVHD, and three other
animals exhibited histologic SGVHD. The lowest dose of irradiation, 300 rad, was
ineffective in abolishing the resistance to the adoptive transfer of disease. Similarly,
infusion of effector splenocytes into nonirradiated animals did not transfer the dis-
ease (data not shown). Decreasing doses of radiation correlated with a reduction
in the histologic grade, hence severity, of acute GVHD. Secondary recipients treated
with 1,050 or 750 rad demonstrated predominately a grade three acute SGVHD
histologically. The animal with clinical SGVHD treated with 500 rad demonstrated
a histologic grade 2.

The Role of Other Preparative Regimens in the Induction versus Adoptive Transfer of Autoreac-
tivity. As indicated above, irradiation is an integral component in both generating
autoimmune precursors and abolishing host resistance to adoptive transfer; other
preparative regimens were the next tools in which to distinguish the individual char-
acteristics inherent in each system using an experimental design similar to that de-
scribed above for defining the radiation sensitivity. CY at all doses, 200, 100, 50,
and 25 mg/kg, combined with a 30-d subsequent regimen of CsA, was an ineffective
preparative regimen for the primary induction of SGVHD, as shown in Fig. 2. However
CY at a dose of 200 or 100 mg/kg given on the day before adoptive transfer resulted
in a maximum incidence of acute SGVHD clinically detectable by day 10. The severity
as noted pathologically was a grade 2 acute SGVHD at a dose of 200 mg/kg and
dropped to a grade 1 acute SGVHD at 100 mg/kg. These data suggest that the
abolishing of host resistance to adoptive transfer of SGVHD is dependent on the
dose of CY used.
Table I demonstrates that a busulfan preparative regimen of 30 mg/kg on day -2 before bone marrow rescue and subsequent CsA therapy did not result in the induction of SGVHD: a low incidence of SGVHD (one of eight rats) did occur with concomitant busulfan and CY therapy, but did so much later (day 28) compared with that observed (day 10-14) when irradiation (1,050 rad) was used as the ablative therapy. In the case of adoptively transferring SGVHD, one of seven rats treated with busulfan treatment alone had a clinical picture of acute SGVHD, which also occurred late. These studies suggest that the nature of the autoregulatory system is radiosensitive, CY sensitive, and relatively busulfan resistant.

Previous studies have shown that thymic irradiation was necessary for the induction of SGVHD (8, 10). We explored whether thymic irradiation was necessary for the adoptive transfer of disease. The data in Table I show that thymic irradiation with a collimated beam focused on the thymus followed by autologous reconstitution and adoptive transfer of effector splenocytes allowed for the successful transfer of SGVHD in five of eight animals. Lower hemibody irradiation did not allow transfer.

The fraction of recipients with clinical GVHD after either inductive therapy or adoptive transfer. The recipients were treated with various preparative regimens. Groups A and B were reconstituted with 50 × 10⁶ bone marrow cells. Groups C and D underwent autologous reconstitution. The rats in the adoptive transfer study received 50 × 10⁶ splenocytes from autoimmune donors.
of SGVHD. These data suggest that thymic irradiation is important for the abolition of host resistance mechanisms specific to this syndrome.

The Characterization of the Autoregulatory Population by Adoptive Cotransfer Studies. The studies above provide evidence that there is a host resistance or autoregulatory system preventing the adoptive transfer of SGVHD. To document and characterize the autoregulatory cell, a series of adoptive cotransfer studies were performed. Two different doses of effector splenocytes from rats with SGVHD were compared since the potencies of a given effector population cannot be predicted. The variables of time after CsA withdrawal, the timing and stage of disease manifestation, or inherent differences in the capability of a population to undergo clonal expansion can explain the varying potencies of effector cells. Both doses of effector splenocytes (15 and \(30 \times 10^6\)) were capable of establishing clinical and grade 2 histologic SGVHD in irradiated secondary recipients (Table II). The transfer of spleen cells from reconstituted non-CsA-treated rats did not mediate disease (data not shown). The cotransfer of \(30 \times 10^6\) unfractionated lymphocytes from a normal rat prevented the establishment of SGVHD in recipients receiving \(15 \times 10^6\) effector lymphocytes and reduced the clinical incidence of SGVHD in those receiving an equivalent dose of effector splenocytes. In both cases the cotransfer of normal unfractionated splenocytes down-regulated the histologic severity of SGVHD from a grade 2 to a grade of 1 of the animals developing disease. If the cotransferred splenocytes were first fractionated over nylon wool and shown to be >85% OX19+ by FACS analysis, then \(30 \times 10^6\) nylon wool-nonadherent splenocytes were as or more effective than unfractionated splenocytes in reducing the incidence of SGVHD upon the cotransfer of an equivalent number of effector splenocytes (Table II, group B). The cotransfer of \(30 \times 10^6\) nylon wool-nonadherent splenocytes and \(15 \times 10^6\) effector splenocytes resulted in

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<td>Group</td>
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The cotransfer of effector splenocytes from autoimmune donors with fractionated and unfractionated splenocytes from a normal animal. Syngeneic marrow (60 \(\times 10^6\) cells) and effector splenocytes (15 \(\times 10^6\) cells in A, 30 \(\times 10^6\) cells in B, and 50 \(\times 10^6\) cells in C) were transferred into lethally irradiated recipients. In A and B, the secondary recipients received either no splenocytes (---), or 30 \(\times 10^6\) unfractionated splenocytes, or 30 \(\times 10^6\) nylon wool-nonadherent (NWNA) splenocytes from a normal animal. In C, the secondary recipients received either no splenocytes, 50 \(\times 10^6\) NWNA splenocytes, or 50 \(\times 10^6\) OX19-depleted NWNA splenocytes from a normal rat.
no evidence of GVHD (Table II, group A) clinically or histologically. Hence nylon wool-nonadherent splenocytes appeared to be more effective in suppressing the transfer of disease than unfractionated splenocytes. Table II, group C, demonstrates that the depletion of nylon wool-nonadherent splenocytes of OX19+ lymphocytes removed the autoregulatory effect present in normal splenocytes. Depletion protocols both by panning and immunomagnetic separation resulted in a T cell-depleted population with <20% staining with W3/13 (another pan-specific T cell marker that has some crossreactivity with other cell types, i.e., polymorphs and stem cells [18]). The transfer of 50 x 10^6 effector splenocytes elicited SGVHD in lethally irradiated secondary recipients: only the cotransfer of nylon wool-adherent splenocytes and not the OX19-depleted population prevented the transfer of disease.

The Critical Period after Transplantation to Initiate CsA Therapy or the Adoptive Transfer of Effector Splenocytes. The present study correlated the time of initiation of daily CsA therapy after irradiation and bone marrow transplantation with the incidence of clinical and pathologically evident SGVHD. A delay in the initiation of CsA therapy for 4–5 d after bone marrow transplantation still resulted in the maximum incidence of SGVHD. If CsA treatment was delayed until 7 d after transplantation, then only 30% of the rats developed SGVHD (Fig. 3).

Likewise, the time of infusion of unfractionated effector splenocytes after lethal irradiation of secondary recipients is critical to elicit a complete transfer of SGVHD (Fig. 4). If effector splenocytes were transferred between day 0 to 4 after transplantation, then all rats manifested severe GVHD usually between 14 to 21 d later. By day 7 after bone marrow transplantation, evidence of a resistance mechanism is clear, since 30% of the secondary recipients were now resistant to the transfer of SGVHD. Rats 14 d after transplantation were comparable to pretransplant rats; both were absolutely refractory to the adoptive transfer of SGVHD.
The pathogenesis of SGVHD remains unclear. This autoaggression syndrome represents a disruption in the normal regeneration of a competent T cell repertoire concurrent with self-tolerance after syngeneic bone marrow transplantation. One of the primary mechanisms thought to account for SGVHD has been the generation of autoreactive cells recognizing self MHC antigens. Recent studies by Hess et al. (12) have shown that the development of SGVHD is associated with the induction of cytotoxic lymphocytes directed against a public epitope of class II antigens. The generation of “self” class II MHC antigen reactive cells appears to be directly related to the effect of CsA on thymic function (11, 19, 20). Previous studies have shown that CsA therapy and irradiation abolishes thymic medullary epithelial cells and causes a reduction in class II antigen expression (11, 19). These irreversible changes are thought to prevent the normal mechanisms of eliminating self-reactive clones. Thymic epithelial cells and dendritic cells are known to be crucial in the education of self-MHC restriction and hence a microinductive environment for self-tolerance. Recent studies have shown that CsA can inhibit the intrathymic events inherent in clonal deletion, by potentially interfering with the T cell receptor signal transduction or reducing class II expression (20). Nevertheless, even in the normal animal, the presence of autoreactive cells in the periphery is evidence of incomplete clonal deletion and implies control by a peripheral regulatory mechanism (21). Therefore, this peripheral mechanism must be eliminated to allow development of SGVHD.

In this study, the disparity between drug and radiation sensitivities exemplifies two distinct underlying mechanisms, the generation of autoreactive cells and the elimination of a host resistance mechanism. First, the radiosensitivity of the host resistance mechanism is more sensitive than that of the inductive mechanism since low doses of irradiation allowed the adoptive transfer of SGVHD but were unable to permit the induction of SGVHD. Secondly, CY in a dose-dependent fashion eliminated host resistance mechanisms but was unable to trigger the events essential for...
induction of SGVHD. Although CY can inhibit peripheral regulatory mechanisms, it was surprisingly ineffective in inducing SGVHD, revealing a possible intrinsic difference between the roles of CY and irradiation in altering intrathymic events essential for disease manifestation. It can also be inferred that the elimination of an autoregulatory mechanism is insufficient by itself for the induction of SGVHD.

The present studies also suggest that resistance to adoptive transfer is a distinct mechanism controlling the development of SGVHD and is consistent with previous studies. While normal nonirradiated rodents upon CsA therapy alone fail to manifest any clinical signs of SGVHD, lymphoid cells from these donors inflict severe autoagression when transferred to a secondary, irradiated recipient (12), implicating a putative autoregulatory mechanism in controlling the induction of SGVHD. Further evidence of an autoregulatory system is demonstrated in the adoptive cotransfer studies. Nylon wool–nonadherent T cells expressing the OX19 determinant, present on mature T lymphocytes, abrogated the transfer and establishment of SGVHD. For every effector splenocyte transferred two unfractionated splenocytes from normal donors were required for inhibition. This evidence demonstrates that an autoregulatory population is present in a normal animal and that the ratio between autosuppressors to autoreactive cells is critical in the inhibition of SGVHD, consistent with the findings of Sorokin et al. (10). Current studies are focused on the analysis of the actual T cell subsets involved.

The delay of CsA administration demonstrated that the initial 4–5 d after transplantation is a critical window in which the initiation of CsA therapy is essential in altering the thymic microinductive environment influencing the generation of self-reactive cells in a class II deficient environment. On the other hand, the ineffectiveness of effector splenocytes that are transferred 1 wk after transplantation to mediate SGVHD demonstrates that the autoregulatory arm is apparently reconstituted within the first week after bone marrow transplantation. Hence lethal irradiation may be critical for abolishing mature suppressor mechanisms present either in the thymus and/or in the periphery. Therefore, the effect of CsA therapy may not only be related to the generation of the effector cell but may also be related to preventing the reconstitution of an autoregulatory mechanism, while allowing the expansion of self-reactive cells.

The synthesis of this data suggests that there are distinct mechanisms involved in disruption of the immunologic balance between autoagression and autoregulatory suppression leading to SGVHD. One facet involves the generation of autoreactive cells; the other involves the disruption of peripherally active tolerance maintaining mechanisms. The generation of self-reactive cells cannot singularly explain SGVHD. In fact autoreactive cells capable of mediating SGVHD can be detected in animals treated with CsA without demonstrating clinical disease when adoptively transferred into a lethally irradiated recipients (11). The prevention of intrathymic clonal deletion and the perturbation of the autoregulatory cell regeneration in a reconstituting animal may be the determining factors in disease manifestation. Abolishment of autoregulation can be the putative event that creates a permissive environment for the expansion of autoreactive cells and the subsequent generation of autoimmune disease. Previous studies suggesting that defects in T cell subsets have been associated with autoimmune disease in experimental animals support this concept (18, 22).
Summary

These studies further delineate the requirements for the establishment and transfer of SGVHD. We show that (a) two mechanisms distinguishable by radiation and drug sensitivities exist, (b) lethal irradiation correlates with a 100% incidence in the induction of SGVHD, whereas (c) both sublethal or lethal irradiation and cytoxan therapy are effective in ablating the host autoregulatory system in order to transfer autoreactivity, (d) unfractionated as well as nylon wool-nonadherent splenocytes effectively inhibit the transfer of autoimmunity, and (e) OX19 depletion of that population, however, destroys the autoregulatory effect present in normal splenocytes. To demonstrate complete inhibition of immune reactivity, twice the number of unfractionated splenocytes from normal animals was required for every splenocyte from autoimmune donors. Last, the infusion of effector splenocytes on 4, 7, and 14 d after transplantation correlates to a decrease from 100%, 70 to 0% incidence of SGVHD, thus emulating the incidence obtained in a pretransplant rat within 2 wk. These findings further clarify the immunobiological complexity of SGVHD and suggest that since autoregulatory cells already exist in normal animals that CsA-induced autoimmunity is a reflection of not an induced reactivity specific to one therapeutic reagent but the uncoupling of normal immunologic mechanisms essential in controlling autoimmunity.

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