Age-Related Factors in Cyclosporine-Induced Syngeneic Graft-Versus-Host Disease: Regulatory Role of Marrow-Derived T Lymphocytes

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Summary

The present studies have evaluated the effect of age on the induction of syngeneic graft-versus-host disease (SGVHD) after syngeneic bone marrow transplantation (BMT) and cyclosporine (CsA) therapy. The results clearly document an inverse correlation of age with the incidence of SGVHD. Virtually a 100% incidence of SGVHD occurs in Lewis rats when syngeneic BMT and CsA therapy are started when the animals are 4 wk of age. Thereafter, there is a dramatic decline in the incidence of SGVHD with the increasing age of the animals. Although the age of the recipient was important, the most significant effect was the age of the marrow donor. Marrow from animals 6 mo of age was virtually incapable of eliciting SGVHD after BMT and CsA therapy. Furthermore, mixing the marrow from mature and immature animals resulted in a decreased incidence of SGVHD, implicating a regulatory effect present in the marrow from older rats. This regulatory effect was due to the presence of mature T cells in the marrow from animals 6 mo of age. Despite the fact that marrow from young animals possesses mature T lymphocytes, this regulatory activity was absent, suggesting that the host resistance mediated by T lymphocytes develops as the animal ages. These data further implicate the importance of a host resistance mechanism in preventing the induction of SGVHD with CsA, which appears to be mediated by the clonal inactivation of autoreactive cells.

Cyclosporine (CsA) is a very potent immunosuppressive agent that has an apparent selective action on T lymphocyte-dependent immune responses (1, 2). It has been used extensively in clinical transplantation to prevent solid organ allograft rejection and graft-versus-host disease (GVHD) (3, 4). CsA is currently being evaluated as a therapeutic agent for the treatment of autoimmune disease, and the results from these trials appear quite promising (5). Despite its potent immunosuppressive activity, CsA therapy after autologous or syngeneic bone marrow transplantation paradoxically elicits a T cell-mediated autoimmune syndrome with pathology identical to GVHD occurring after allogeneic bone marrow transplantation (BMT) (6–10). Hence, this CsA-induced autoimmune syndrome was originally termed syngeneic GVHD (SGVHD) (6). The immunobiology of SGVHD is complex and is not completely understood, but it appears to be due to the uncoupling of normal immunologic homeostasis involved in self-nonself recognition (11).

This autoimmune disease, which occurs 14–21 d after cessation of CsA therapy, is mediated by T lymphocytes (6, 7) and is intimately associated with the appearance of OX8+ (CD8) autoreactive T cells that recognize a public epitope on class II major histocompatibility antigens (8). A W3/25+ (CD4) Th cell subset also plays an essential role in disease manifestation (7). Further studies have suggested that a T lymphocyte-dependent host resistance mechanism must be eliminated before the development of this autoaggressive syndrome can occur (11, 12). Of additional importance are the recent findings that indicate that in addition to thymic irradiation and CsA treatment, age is a critical variable for the induction of SGVHD (13); animals >3 mo of age are much more resistant to the induction of autoimmunity with CsA. The mechanism accounting for the resistance of mature animals is unknown, but it may be related to the lack of a pronounced effect of CsA on the thymic medulla, as postulated by Beschorner et al. (14). On the other hand, recent evidence suggests that there are increased numbers of autoregulatory cells in older animals, thus preventing the clonal amplification of autoreactive cells (13).

In the present study, we demonstrate that there is a significant correlation of age with the induction of SGVHD;
Materials and Methods

Rats. Lewis female (RT') rats, which were corona virus free and 1, 3, or 6 mo of age, or neonatally thymectomized Lewis female rats, were purchased from Charles River Breeding Laboratories, (Wilmington, MA) and kept in sterile microisolator cages.

Total Body Irradiation. Lewis rats were irradiated on day -1 with 1,050 rads at 108 rads/min from a dual source Cs-137 Cs small animal irradiator (Atomic Energy of Canada Ltd., Kanata, Ontario, Canada).

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 cellswere killed by C0 2 asphyxiation. Marrow was collected from the back and end of the tailvein on day 0. The total volume injected was 1 ml.

Antibiotics. Rats received medicated drinking water supplemented with bacitracin, neomycin, and polymyxin B, as previously described (6).

CsA. 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/kg subcutaneously from the day of marrow infusion for 30 consecutive days. The total volume injected was 1 ml.

Assessment of SGVHD. Rats were examined daily for signs of clinical GVHD. Ear biopsies were taken at frequent intervals. Initial onset of SGVHD occurred within 7-14 d after discontinuation of CsA therapy and was presented as an acute GVHD with erythema and dermatitis (9). Within 2 wk after onset, the disease progressed to a more chronic type of GVHD with alopecia and fibrosis (9). Animals were followed 6-8 wk after discontinuation of CsA therapy to assess either a delayed appearance of disease, resolution of disease, or progression and development of extensive disease.

Statistical Analysis. Results were analyzed by multivariate analysis or by the chi2 test.

Results

The Effect of Age on the Induction of SGVHD. Initial studies were undertaken to assess the effect of age on the induction of SGVHD in Lewis rats treated with CsA after syngeneic marrow transplants. Animals of 1, 3, or 6 mo of age were irradiated, reconstituted with marrow from similarly aged
syngeneic donors, respectively, and treated with graded doses of CsA for 30 d. The results in Fig. 1 demonstrate a significant effect of age on the induction of SGVHD. 1-mo-old transplanted Lewis rats demonstrated a virtual 100% incidence of SGVHD when treated with 10 and 15 mg of CsA; a 70% incidence was observed when the animals were treated with 5 mg/kg of this drug. In contrast, a moderate incidence (50%) of SGVHD was observed in animals that were transplanted at 3 mo of age, and only at the highest dose of CsA tested. A minimal incidence of SGVHD was observed in rats that were initially transplanted at 6 mo of age.

A series of experiments was performed to further analyze the effect of age on the induction of SGVHD by separating marrow donors and recipients on the basis of age. The study was designed to assess whether donor and/or host age is a factor that influences the frequency of SGVHD induced with CsA. Lewis rats of either 1, 3, or 6 mo of age were used as donors of bone marrow for lethally irradiated 10-wk-old recipients. The reciprocal experiment was performed in which the 1-, 3-, and 6-mo-old Lewis rats served as recipients of bone marrow from 10-wk-old rats. The recipients were treated with graded doses of CsA for 30 d after irradiation and bone marrow reconstitution. Fig. 2 A illustrates that there was a significant \( p < 2 \times 10^{-4} \) correlation of the age of the marrow donor with the induction of SGVHD in 10-wk-old recipients. The greatest frequency of SGVHD was observed in animals grafted with marrow from rats 1 mo of age. Marrow derived from animals 3 or 6 mo of age resulted in a decreased incidence of SGVHD. Comparatively, the age of recipient also correlated \( p < 10^{-3} \) with the ability of CsA to induce SGVHD. The youngest recipients had the greatest incidence of SGVHD. The effect of the dose of CsA was more intimately associated with donor age than with recipient age. However, lowering the doses of CsA administered in the recipient study (Fig. 2 B) was reflected in a less dramatic decline in incidence.

Additional studies summarized in Fig. 3 further assessed the combined effects of donor and recipient age on the induction of SGVHD. Lewis rats 1 mo of age were used as donors of bone marrow for irradiated syngeneic recipients that were 10 wk of age. The recipients were treated with graded doses of CsA for 30 d. Rats 6 mo of age received bone marrow from 10-wk-old syngeneic donors and treated with graded doses of CsA for 30 d. The recipients (A and B) were observed for the appearance of SGVHD after discontinuation of CsA treatment \( (n = 10 \) per group).
the combined effects of donor and recipient age at a 15 mg/kg dose of CsA using marrow donors and recipients of either 1 or 3–6 mo of age. The results revealed that the greatest incidence of SGVHD after CsA therapy occurred when the marrow was derived from donors 1 mo of age. At this dose of CsA, SGVHD was induced in 90% of rats 3–6 mo of age when the marrow donor was 1 mo old. Taken together with the results presented in Fig. 2B, it appears that the recipient age variable can be overcome by increasing the dose of CsA and the use of donor marrow derived from younger animals. In contrast, when marrow is derived from donors that are ≥3 mo of age, only a moderate (30%) incidence of SGVHD occurs after CsA therapy, and it was not enhanced significantly by increasing the dose of CsA.

The Effect of Combining Unfractionated Marrow from Young and Old Donors on the Incidence of Syngeneic GVHD The next series of experiments were designed to address whether mixing unfractionated marrow from animals 1 and 6 mo of age could modify the incidence of syngeneic GVHD normally induced with bone marrow from the youngest donors alone.

Bone marrow from donors 1 or ≥6 mo of age was transfused at first alone into lethally irradiated 8–9-wk-old recipients. As controls, two different doses, 3 × 10^7 and 6 × 10^7 nucleated cells, were used to reconstitute the recipients. The experimental groups received a total of 6 × 10^7 bone marrow cells derived from donors 1 and ≥6 mo of age mixed in equal proportion. The recipients received 30 d of CsA therapy (15 mg/kg/d), and the incidence of syngeneic GVHD was observed. The results are summarized in Table 1. Recipients of bone marrow from donors 1 mo of age demonstrated severe disease in the entire experimental group by at least day 28 after discontinuation of CsA therapy. Those that received 6 × 10^7 developed a more aggressive disease 3 wk earlier than those receiving 3 × 10^7 cells. Notably, recipients of marrow from donors ≥6 mo of age did not develop disease if reconstituted with 3 × 10^7 cells and only one of eight rats transfused with 6 × 10^7 cells developed mild SGVHD. Recipients of 3 × 10^7 cells from 1-mo-old donors combined with 3 × 10^7 cells from donors ≥6 mo of age demonstrated the same timing and incidence of clinical SGVHD as those receiving marrow from the mature donors alone. In summary, the transfusion of marrow from more mature animals influences the potential of bone marrow from immature animals to induce autoimmunity. The marrow from donors ≥6 mo of age contained some population(s) of cells capable of preventing the induction of syngeneic GVHD in the secondary recipient.

| Table 1. The Effect of Mixing Unfractionated Marrow from Animals 1 and 6 mo of Age on the Induction of Syngeneic GVHD |
|---------------------------------------------------------------|
| **Marrow graft composition** | **Incidence and severity of SGVHD** |
| **Age of marrow donor** | **Cell dose** | **Fraction with clinical SGVHD** | **Day 7** | **Day 28** | **Severity** |
| | | | | | |
| × 10^7 | |
| 6 mo | 3 | 0/6 | 0/6 | Mild/recovered |
| | 6 | 0/8 | 1/8 |
| 1 mo | 3 | 0/8 | 8/8 | Severe/died |
| | 6 | 8/8 | 8/8 |
| 6 + 1 mo | 3 | 0/8 | 1/8 | MILD/recovered |
| | 3 |

Irradiated Lewis recipients (8–9 wk of age) were grafted with syngeneic bone marrow derived from syngeneic rats 1 mo of age or from animals 6 mo old. The marrow was infused singly or after mixing, and the recipients were treated with a 30-d course of CsA (15 mg/kg/d). After discontinuation of CsA therapy, the animals were observed daily for the appearance of SGVHD.
Table 2. The Effect of Mixing Unfractionated Marrow from Animals 1 mo of Age with Elutriated Marrow from Animals 6 mo of Age on the induction of Syngeneic GVHD

| Marrow graft composition | Incidence of SGVHD |
|--------------------------|--------------------|
| Marrow cell dose from animals 1 mo of age | Marrow fraction from animals 6 mo of age (4 x 10^7 cells) | (fraction with clinical SGVHD) |
| x 10^7 | None | 8/8 |
| 4 | Unfractionated | 5/8 |
| 4 | 25 | 0/8 |
| 4 | 29 | 5/8 |
| 4 | R/O | 7/8 |
| 2 | None | 8/8 |
| 2 | Unfractionated | 3/8 |
| 2 | 25 | 0/8 |
| 2 | 29 | 6/7 |
| 2 | R/O | 6/7 |
| 0 | Unfractionated | 1/8 |
| 0 | 25 | Failed to Engraft |
| 0 | 29 | 3/4 |
| 0 | R/O | 4/4 |

Marrow was harvested from 6-mo-old Lewis rats and fractionated by elutriation. Unfractionated marrow or the respective 25, 29, R/O elutriation fractions (4 x 10^7 cells) were added to marrow derived from 1-mo-old syngeneic Lewis rats and infused into Lewis rats (8-9 wk of age). As controls, the recipients received either marrow from rats 1 or 6 mo of age or the elutriation fractions separately. The recipients were treated with a 30-d course of CsA (15 mg/kg/d) and observed for the onset of SGVHD.

Marrow from donors 6 mo of age resulted in five of eight rats developing SGVHD. However, SGVHD did not occur in recipients receiving either 2 or 4 x 10^7 bone marrow cells from the immature donors and 4 x 10^7 elutriated bone marrow cells isolated in fraction 25 from donors 6 mo of age. Thus, fraction 25 had the most potent inhibitory effect on the prevention of syngeneic GVHD. Addition of the R/O and/or fraction 29 from marrow of 6-mo-old animals to unfractionated marrow from donors 1 mo of age did not significantly influence the induction of syngeneic GVHD. Furthermore, animals grafted separately with the R/O fraction or the 29 fraction from marrow of animals 6 mo of age, engrafted and developed SGVHD after a course of CsA (15 mg/kg) therapy. Animals did not engraft when fraction 25 was used singly.

The potent capability of unfractionated marrow from donors 6 mo of age to inhibit the primary induction of syngeneic GVHD with marrow from immature animals appeared to be isolated in elutriation fraction 25. This fraction also accounted for the inability of marrow from animals 6 mo of age to allow the induction of SGVHD. Further studies were undertaken to phenotypically characterize the cells contained in fraction 25 of marrow from both mature (6 mo) and immature (1 mo) Lewis rats. Single-color flow cytometric analysis demonstrated that fraction 25 from animals 6 mo of age (mean of three experiments) had a predominance of cells with mature T lymphocyte markers: 22.6% OX19^+, 12.3% CD4^+, and 8.4% CD8^+. (Table 3). Fraction 25 from 1-mo-old animals had 12.5% cells with mature T cell markers, and a predominance of B cells (OX 33^+) and cells expressing OX7 or Thy-1.1 (B cells also express OX7). There was also a significant percentage of cells not staining with any markers. The greatest difference appeared to be the reduced number of T cells in fraction 25. Comparatively, the marrow R/O and 29 fractions from both groups had minimal total T cells defined by staining with OX19 (fraction 29 <15%; R/O <5%). Furthermore, fraction 29 is only a small percentage (11-16%) of the entire elutriated marrow.

Experiments were undertaken to assess if depletion of the mature T cells or the cells expressing the OX7 marker in fraction 25 of marrow from mature Lewis rats removed the ability of this fraction to inhibit induction of SGVHD. Irradiated Lewis rats (8-10 wk of age) were grafted with marrow from donors 1 mo of age to which unfractionated marrow, fraction 25, and fraction 25 depleted of T cells or OX7^+ cells was added (derived from animals 6 mo of age). The recipient animals were treated with the standard course of CsA (30 d, 15 mg/kg/d), and the incidence of SGVHD was observed after withdrawal of CsA therapy. The results in Table 4 show that only depletion of the mature T lymphocytes removed the inhibitory activity of fraction 25 on the induction of SGVHD. Further studies showed that depletion of either the W3/25 (CD4) or OX8 (CD8) subsets alone removed the inhibitory effect on the induction of SGVHD. However, recombining the fractions restored the inhibitory activity as reflected by the prevention of SGVHD, indicating that both subsets were required.

Marrow from Lewis rats 1 mo of age also contained mature T cells, but yet allowed for induction of SGVHD. We

Table 3. Phenotypic Analysis of Elutriation Fraction 25 from Marrow Donors 1 and 6 mo of Age

| Donor | Age of marrow donor |
|-------|---------------------|
| OX 19 (T lymphocytes) | 12.5 | 22.6 |
| CD4 | 6.3 | 12.3 |
| CD8 | 5.2 | 8.4 |
| OX 33 (B lymphocytes) | 30.1 | 11.8 |
| OX 7 (Thy-1.1) | 71.6 | 26.1 |

Marrow derived from immature (1 mo) and mature (6 mo) Lewis rats was elutriated, and the cells in fraction 25 were assessed for expression of known phenotypic markers by single-color flow cytometric analysis. Results are the mean of three separate experiments.
Table 4. The Effect of T Cell Depletion of Marrow Fraction 25 from Mature Donors on the Induction of SGVHD

| Age of marrow donors | Unfractionated marrow | Fraction of marrow from animals 6 mo of age added (4 × 10^7) | Incidence of SGVHD (fraction with clinical SGVHD) |
|----------------------|-----------------------|-------------------------------------------------------------|--------------------------------------------------|
| 1 mo                 | × 10^7                |                                                             |                                                  |
| 4                    | –                     |                                                             | 8/8                                              |
| 3                    | –                     |                                                             | 9/9                                              |
| 3                    | Unfractionated marrow |                                                             | 0/6                                              |
| 3                    | FR 25                 |                                                             | 0/8                                              |
| 3                    | FR 25: Ox7 depleted   |                                                             | 0/4                                              |
| 3                    | FR 25: T cell depleted|                                                             | 0/4                                              |
| 4                    | FR 25: CD8 depleted   |                                                             | 4/4                                              |
| 4                    | FR 25: CD4 depleted   |                                                             | 4/4                                              |
| 4                    | FR 25: CD8 depleted + |                                                             | 0/3                                              |
| 6 mo                 | × 10^7                |                                                             | 0/3                                              |

Marrow was harvested from Lewis rats 6 mo of age and fractionated by elutriation. Fraction 25 or the lymphocyte-enriched fraction was depleted of OX7+ cells or T lymphocytes (OX19, OX8, W3/25 cocktail) with immunomagnetic beads. The unfractionated marrow, fraction 25, and/or the depleted fractions were mixed with marrow derived from syngeneic rats 1 mo of age and grafted into irradiated Lewis recipients (6–8 wk of age). The animals were treated with CsA (15 mg/kg/d, for 30 d) and observed for the onset of SGVHD after discontinuation of therapy.

attempted to assess if the ability of young marrow to allow induction of SGVHD was due to the decreased number of mature T cells described above or due to an absence of specific regulatory cells that reside in fraction 25 of marrow from older animals. A dose-response study was performed in which graded doses of T lymphocytes in fraction 25 of marrow from both Lewis rats 1 and 6 mo of age were infused with unfractionated marrow from 1-mo-old animals into irradiated recipients (6–8 wk of age). The recipients were treated with CsA (15 mg/kg/d) for 30 d, and the incidence of SGVHD was observed. The results in Table 5 demonstrate that addition of ≥5 × 10^6 T cells from fraction 25 of donors 6 mo of age completely abolished the induction of SGVHD, while 2.5 × 10^6 T cells reduced the incidence of SGVHD to 50%. Comparatively, even the addition of 10^7 T lymphocytes of fraction 25 from the marrow of animals 1 mo of age did not inhibit the induction of SGVHD. Similarly addition of splenic (nylon wool–nonadherent) T lymphocytes (≥10^7) from animals 6 mo of age to the inoculum of marrow, prevented the development of SGVHD after CsA therapy. On the other hand, only the highest dose of splenic T lymphocytes from donors 1 mo of age inhibited the induction of SGVHD.

Role of the Host Resistance Mechanism on the Induction of SGVHD. A series of experiments was undertaken to identify the role of the host resistance mechanism in preventing the induction of SGVHD. The results from the studies described above indicate that addition of mature T lymphocytes from animals 6 mo of age to the marrow inoculum at the time of transplant prevented the development of SGVHD. One hypothesis to account for the inhibition of the development of SGVHD is that the mature T lymphocytes (contained in marrow or in spleen) expand during CsA therapy to levels capable of preventing the expression of the autoreactive cells generated during therapy. Recent data suggest that mature T lymphocytes can expand in a thymic- and antigen-independent environment (18). To assess if the mature T lymphocytes including the cells responsible for host resistance clonally expand, thymectomized Lewis rats were grafted with marrow depleted of T lymphocytes by elutriation. 2 wk after transplantation, the recipients were infused with 5 × 10^6 splenic T lymphocytes (nylon wool–nonadherent cells) and treated with CsA (10 mg/kg/d) or the control diluent for 30 d. After therapy, the recipients were infused with 3 × 10^7 effector T lymphocytes from animals with active SGVHD. The results in Table 6 demonstrate that SGVHD could not be transferred into the bone marrow–reconstituted, thymectomized recipients grafted with 5 × 10^6 T lymphocytes. However, SGVHD could be transferred if the recipients were treated with CsA. This was not due to the primary induction of SGVHD, since control thymectomized recipients treated with CsA did not develop disease, thus confirming the data of Sorokin et al. (7). These data suggest that CsA interferes with the expansion of a T lymphocyte–dependent host resistance mechanism carried over with mature T lymphocytes and could not prevent the adoptive transfer of disease with 3 × 10^7 effector cells.
Table 5. Efficacy of T Lymphocytes in Marrow Fraction 25 and Spleen to Inhibit the Induction of SGVHD

| Age of marrow donors and graft composition | Incidence of SGVHD (fraction with clinical SGVHD) |
|-------------------------------------------|--------------------------------------------------|
| Marrow donors (1 mo of age)               |                                                  |
| Source and number of T cells added        |                                                  |
| × 10⁷                                     |                                                  |
| Marrow (6 mo)                             | × 10⁴                                            |
| 4                                         | × 10⁴                                            |
| 4                                         | Fraction 25 (10⁷)                                |
| 4                                         | Fraction 25 (5 × 10⁶)                             |
| 4                                         | Fraction 25 (2.5 × 10⁶)                           |
| × 10⁷                                     |                                                  |
| Marrow (1 mo)                             | × 10⁴                                            |
| 4                                         | × 10⁴                                            |
| 4                                         | Fraction 25 (10⁷)                                |
| 4                                         | Fraction 25 (5 × 10⁶)                             |
| 4                                         | Fraction 25 (2.5 × 10⁶)                           |
| Spleen (6 mo)                             | × 10⁴                                            |
| 4                                         | × 10⁴                                            |
| 4                                         | 3 × 10⁷                                          |
| 4                                         | 10⁷                                              |
| Spleen (1 mo)                             | × 10⁴                                            |
| 4                                         | × 10⁴                                            |
| 4                                         | 3 × 10⁷                                          |
| 4                                         | 10⁷                                              |

Graded doses of T lymphocytes in fraction 25 of elutriated marrow and in nylon wool–nonadherent spleen cells derived from Lewis rats of 1 and 6 mo of age were added to marrow of 1-mo-old syngeneic animals. The number of T cells was estimated by expression of OX19 with immunofluorescent staining. The mixture of cells was infused into irradiated syngeneic Lewis recipients (6–8 wk of age), and the rats were treated with the standard course of CsA therapy (15 mg/kg/d, for 30 d). Animals were observed for the onset of SGVHD after discontinuation of CsA treatment.

Table 6. Effect of CsA on the Reconstitution of Host Resistance by Peripheral T Lymphocytes

| Treatment of thymectomized recipients | Marrow | T cells | CsA therapy (10 mg/kg for 30 d) | Infusion of SGVHD effector cells (3 × 10⁷) on day of CsA discontinuation | Incidence of SGVHD |
|---------------------------------------|--------|---------|---------------------------------|------------------------------------------------------------------------|-------------------|
| × 10⁷                                 | × 10⁴  |         |                                |                                                                        |                   |
| R/O (4)                               | 5      | +       |                                |                                                                        | 4/4               |
| R/O (4)                               | 5      | -       |                                |                                                                        | 0/4               |
| R/O (4)                               | -      | +       |                                |                                                                        | 4/4               |
| R/O (4)                               | -      | -       |                                |                                                                        | 4/4               |
| R/O (4)                               | 5      | +       |                                |                                                                        | 0/4               |
| R/O (4)                               | 5      | -       |                                |                                                                        | 0/4               |

Thymectomized Lewis rats were irradiated (1,050 rad) and reconstituted with syngeneic marrow depleted of T lymphocytes by elutriation. 2 wk after transplant, the recipients were infused with 5 × 10⁶ splenic T lymphocytes from rats 6 mo of age, and the animals were treated with CsA or the control diluent for 30 d. On day 30, 3 × 10⁷ effector cells from animals with active SGVHD were adoptively transferred into the thymectomized recipients.

Discussion

The immunobiological mechanisms accounting for the induction of SGVHD by administration of CsA after syngeneic BMT are indeed complex and remain enigmatic. Central to
Table 7. Effect of T Lymphocytes Infused on the Day of Transplant or After CsA Therapy on the Induction of SGVHD

| Transfer of T lymphocytes | Incidence of SGVHD |
|--------------------------|-------------------|
| Day of transfer          | No. of cells transferred | Incidence of SGVHD |
|                          | × 10⁶               |                      |
| Day 0: day of transplant | 3                  | 0/4                  |
|                          | 2                  | 0/4                  |
|                          | 1                  | 0/4                  |
| Day 30: discontinuation  | 3                  | 3/4                  |
| of CsA therapy           | 2                  | 4/4                  |
|                          | 1                  | 4/4                  |

Graded doses of splenic T lymphocytes from animals 6 mo of age were adoptively transferred into bone marrow (derived from animals 1 mo of age)-reconstituted syngeneic Lewis recipients on the day of transplant or on the last day of a 30-d course of CsA therapy.

The effect of the recipient's age on the induction of SGVHD is listed in Table 7. The most surprising finding of our studies was that the incidence of SGVHD was significantly associated with the age of the marrow donor and had an even greater effect than the age of the recipient. In addition, the age of the recipient became less important if the donor marrow was derived from the youngest animals. Marrow from animals 6 mo of age was, to a large extent, incapable of permitting the induction of SGVHD despite the age of the recipient. In contrast, SGVHD could be consistently induced, even in mature animals, provided that the marrow was derived from animals 4 wk of age. Furthermore, mixing marrow from donors 1 and 6 mo of age elicited a reduced incidence of SGVHD, mimicking that obtained with marrow derived from rats 6 mo of age. These data implicate a regulatory effect present in the marrow of mature animals. Further studies demonstrated that the regulatory effect in the marrow from animals 6 mo of age was due to the presence of small, mature T lym-
phocytes. Depletion of the T lymphocytes eliminated the modifying influence of marrow derived from mature animals on the induction of SGVHD when co-infused with marrow from rats 1 mo of age. In further support of the regulatory effect of mature T lymphocytes are the findings that: (a) T cell-depleted marrow from mature animals was able to permit the development of SGVHD; and (b) addition of splenic T lymphocytes from 6-mo-old rats to the marrow from animals 1 mo old prevented the induction of SGVHD. In contrast, although slightly less in number, the mature T lymphocytes contained in marrow from the immature animals did not prevent the development of SGVHD. Dose-response studies clearly suggested that this regulatory activity was absent in marrow from the immature animals or was present at a very low frequency. Comparatively, some regulatory activity of splenic T lymphocytes from young animals on the induction of SGVHD was observed, but only at higher concentrations of cells. Much lower numbers of splenic T lymphocytes from animals 6 mo of age were required to prevent the induction of SGVHD. Taken together, these data suggest that this T cell-dependent regulatory system capable of modifying the induction of SGVHD develops as the animal matures. Our data are consistent with the observations of Sakaguchi and Sakaguchi (13), who demonstrated that administration of CsA to newborn mice induced pleomorphic organ-specific autoimmune diseases. CsA treatment was also noted to be far more effective if started on the day of birth rather than the third to seventh day after birth. Not even higher doses or protracted periods of CsA treatment could induce autoimmunity in adult mice. Sakaguchi and Sakaguchi (13) attributed these effects to the development of an autoregulatory system that prevented the amplification and expression of autoregulatory clones. They also postulated that CsA prevented the development of this autoregulatory system and that a temporary absence of this thymic-dependent regulatory system allowed the differentiation/activation of autospecific effector cells.

Some of the early studies clearly implicated the role of a host resistance mechanism involved in controlling CsA-induced SGVHD. The primary evidence included the finding that SGVHD could only be transferred into irradiated secondary recipients, not into normal animals, and that CsA treatment of normal animals did not induce the SGVHD syndrome although adoptive transfer of these spleen cells into irradiated secondary recipients resulted in the development of syngeneic GVHD (6, 10, 22). These findings suggest that normal animals possess a radiation-sensitive component that regulates the activity of autoreactive cells, thus preventing the development of autoimmune GVHD. Adoptive transfer studies in our laboratory provided evidence that an irradiation- and cyclophosphamide-sensitive thymic-dependent system played a major role in the prevention of SGVHD (11, 12). Furthermore, normal splenic T lymphocytes, when cotransferred with autoinimmune effector cells, prevented the development of syngeneic GVHD in secondary recipients (12). This regulatory effect of normal splenic T lymphocytes required collaboration between CD4+ and CD8+ T cell subsets, findings comparable with the results of the present studies. Of importance was the finding that the adoptive transfer of this host resistance mechanism was dose dependent, requiring twice the number of splenic T lymphocytes to SGVHD effector cells. In contrast, the present studies demonstrate that addition of small numbers of T lymphocytes to the marrow graft at the initiation of the induction phase prevented the development of SGVHD. The mechanism whereby the T lymphocytes from mature animals inhibit the induction of SGVHD is unclear. One possibility is that the mature T lymphocytes contained in the marrow graft expand (despite the presence of CsA) to levels that are able to modify the action of the autoreactive cells produced during the 30-d course of CsA therapy. Recent evidence presented by Powrie and Mason (18) suggest that mature T cells can proliferate in a thymic- and antigen-independent environment (thymectomized hosts reconstituted with T cell-depleted marrow). The present studies demonstrate that thymectomized rats, reconstituted with T cell-depleted marrow and infused with small numbers of T lymphocytes were resistant to the adoptive transfer of SGVHD with effector splenocytes after waiting 30 d to allow expansion of the normal T cells. However, administration of CsA during this 30-d period prevented the expansion of this host resistance mechanism, and SGVHD was successfully transferred, clearly indicating that CsA prevented the expansion of this host resistance mechanism such that a limited number of effector cells (3 x 10^6) were capable of establishing disease in these animals. Further, infusion of 3 x 10^7 normal T lymphocytes on the day of CsA withdrawal failed to prevent development of SGVHD. These data imply that the number of autoreactive cells generated during CsA therapy exceeded the capacity of the host resistance mechanism contained in 3 x 10^7 normal T lymphocytes. On the other hand, only a minimum number of normal T cells (5 x 10^5) were required to be added to the marrow inoculum and infused on the day of transplant, to prevent the development of SGVHD. Since CsA administration prevented the expansion of the host resistance mechanism, it would seem likely that the host resistance mechanism contained in the mature T lymphocyte population carried over with the marrow graft inactivates the autoreactive cells as they are generated during the course of CsA therapy. This hypothesis is also supported by the failure to adoptively transfer SGVHD from animals grafted with marrow plus mature T lymphocytes to irradiated secondary recipients in which the host resistance mechanism had been eliminated. If the failure to induce SGVHD in animals grafted with marrow plus T lymphocytes from mature animals was due to a delicate balance of autoreactivity and host resistance, thus maintaining active control (suppression) of the autoreactive cells, one would have expected to be able to transfer disease in an environment of reduced or absent host resistance, as shown by Cheney and Sprent (10). Taken together, it seems likely that host resistance mediated by T lymphocytes from mature animals clonally inactivate the autoreactive cells. Inactivation or induction of clonal anergy is thought to be the major mechanism controlling autoreactive cells that have escaped clonal deletion mechanisms in the thymus (26).
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