GRAFT-VERSUS-HOST DISEASE IN CYCLOSPORIN A-TREATED RATS AFTER SYNGENEIC AND AUTOLOGOUS BONE MARROW RECONSTITUTION

BY ARNOLD GLAZIER,* PETER J. TUTSCHKA, EVAN R. FARMER, AND GEORGE W. SANTOS

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

Cyclosporin A (CsA) is a potent reversible immunosuppressive agent which is able to suppress allograft rejection and acute allogeneic graft-versus-host disease (GVHD) (1–7). Although CsA dramatically improves survival after allogeneic marrow transplantation, rats treated with a short course of CsA develop delayed onset GVHD after CsA withdrawal. This paper presents new evidence which indicates that GVHD may develop upon CsA withdrawal following not only allogeneic but also syngeneic and even autologous bone marrow reconstitution.

Materials and Methods

Rats. Lewis female RT1 rats, 6–8 wk old, were purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN.

Radiation. Lewis rats were irradiated on day −1 (1,020 rad) at 120 rad/min from a dual source Cs137 small animal irradiator (Atomic Energy of Canada Ltd., Ottawa, Ontario). Leg-shielded rats were then anesthetized with chloral hydrate, their right tibias shielded with a lead doughnut 1.5 cm thick and 2.5 cm long, and irradiated with 1,020 rad.

Marrow Transplantation. Donor marrow from tibias, femurs, and humeri was suspended at 6 × 106 nucleated cells/ml in Hanks' solution supplemented with 50 U/ml penicillin and 50 ng/ml streptomycin. On day 0 rats received 1 ml via the tail vein.

Antibiotics. Rats received medicated drinking water supplemented with bactrim, neomycin, and polymyxin B and were given 2.5 mg/kg gentamycin per day subcutaneously for 20 d. Rats that developed overt clinical infections were routinely sacrificed and excluded from the study.

Cyclosporin A. CsA was the generous gift of Sandoz, Inc., Hanover, NJ. Emulphor EL-620 was obtained from GAF Corp., New York. Powdered CsA was dissolved in absolute ethanol, added to emulphor, and mixed with water to yield a final emulphor concentration of 5%. Rats were weighed daily and received 1 ml/100 g body weight per day subcutaneously from day 0 to 40 or as indicated.

Assessment of GVHD. Rats were examined daily for signs of clinical GVHD such as red ears, dermatitis, or diarrhea. Skin biopsies were taken at frequent intervals. When rats developed severe clinical GVHD characterized by weight loss, hunched appearance, and extensive dermatitis they were sacrificed and autopsied. Previously described criteria were

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To whom reprint requests should be addressed at The Johns Hopkins Oncology Center 3-127, 600 North Wolfe Street, Baltimore, MD 21205.

Abbreviations used in this paper: CsA, cyclosporin A; GVHD, graft-versus-host disease.
FIGURE 1. Grade II acute GVHD in a CsA-treated rat after autologous reconstitution. Note extensive mononuclear epidermal infiltrate. × 510.

FIGURE 2. Syngeneic GVHD in the tongue of a CsA-treated Lewis chimera. There is a mucosal and submucosal mononuclear infiltrate with islands of mucosal destruction. × 510.
Figure 3. Esophagus from a rat with syngeneic GVHD with extensive lymphocyte exocytosis and destruction of basal mucosa. × 310.

Figure 4. Hepatic syngeneic GVHD. Note extensive mononuclear infiltrate in portal triad. × 310.
Table I

GVHD in Cyclosporin A-treated Syngeneic Chimeras

| CsA dose (mg/kg/d) | Days of treatment | Fraction with grade II GVHD |
|-------------------|-------------------|-----------------------------|
| 0                 | 20                | 1/64*                       |
| 2.5               | 20                | 0/3                         |
| 7.5               | 20                | 8/15                        |
| 7.5               | 40                | 21/22                       |
| 15                | 20                | 9/14                        |
| 15                | 40                | 17/20                       |
| 25                | 20                | 3/14                        |
| 25                | 40                | 8/8                         |
| 40                | 20                | 2/6                         |

Lethally irradiated Lewis rats were reconstituted with syngeneic marrow and treated with CsA at the dose indicated for either 20 or 40 d beginning on day 0. Results pooled from three experiments.

* Control group represents data pooled from seven experiments.

used for the histological documentation of GVHD (8). Grade II acute GVHD was defined by the presence of lymphocytic exocytosis, epidermal destruction with vacuolar changes of the basal layer, and dyskeratotic cells.

Adoptive Transfer of GVHD. Donor spleens were suspended in Hanks' solution by gently pressing the spleens through a stainless steel screen. GVHD was adoptively transferred with $6 \times 10^7$ marrow cells and $10^8$ spleen cells given intravenously to irradiated syngeneic rats. In some experiments marrow and spleen cells were pooled from donors with GVHD.

Results

Syngeneic GVHD. Lethally irradiated Lewis rats were reconstituted with syngeneic bone marrow and treated with control diluent or CsA. Syngeneic radiation chimeras not treated with CsA as a rule did not develop clinical or histological GVHD. In seven independent syngeneic transplants only 1 of 64 chimeras not treated with CsA developed GVHD. At autopsy this rat had intense sialodacroadenitis suggestive of an underlying coronavirus infection. In contrast 68 of 97 rats reconstituted with syngeneic marrow and treated with CsA at 7.5–40 mg/kg per day for 20–40 d developed clinical and histological GVHD ($P < 0.001$) (Table I). GVHD was not seen while the rats were receiving CsA. Erythematous ears, dermatitis, hair loss, diarrhea, and weight loss were observed in most rats 12–40 d after the CsA was stopped. Characteristic histological lesions of GVHD were seen in the skin, tongue, esophagus, and liver (Fig. 1–4). Pronounced lymphoid atrophy of the thymus, spleen, and lymph nodes was observed in syngeneic chimeras that developed GVHD after a 20-d course of CsA. Lymphoid atrophy was less striking in rats which developed GVHD after 40 d of post-transplant CsA treatment. Large numbers of lymphoblasts were observed in the lymph nodes of these rats at day 55.

Autologous GVHD. It might be argued that CsA merely amplifies a GVHD response due to subtle genetic differences between "syngeneic rats". To test this hypothesis Lewis rats were irradiated with 1,020 rad, shielding the right tibia with a lead doughnut. Four out of four leg-shielded CsA-treated rats developed clinical and histological acute GVHD. We have observed no GVHD in seven
untreated leg-shielded rats (Table II).

Adoptive Transfer of Syngeneic and Autologous GVHD. Table III shows the results of four independent adoptive transfer experiments. Syngeneic GVHD may be adoptively transferred with spleen cells and marrow to lethally irradiated syngeneic recipients. Unirradiated rats are resistant to the adoptive transfer of syngeneic GVHD. Preliminary results suggest that both syngeneic and autologous GVHD is mediated by T cells. The adoptive transfer of both syngeneic and autologous GVHD was prevented by treating the spleen cells with a potent rabbit anti-rat thymocyte serum (9) and guinea pig complement. Complement alone did not interfere with the adoptive transfer of the GVHD.

Normal rats are refractory to the adoptive transfer of syngeneic GVHD and normal untransplanted rats do not develop GVHD after CsA withdrawal (data not shown).

These results suggest that normal rats possess active tolerance-maintaining mechanisms that prevent the development of autologous GVHD and the transfer of established syngeneic GVHD. Table IV shows the results of an adoptive transfer experiment in which lethally irradiated Lewis rats were transplanted with Lewis marrow and syngeneic spleen cells from rats with GVHD. One group

| Table II |
|----------|
| Autologous GVHD |
| Treatment | Fraction with histologically verified clinical GVHD |
| No CsA | 0/7 |
| CsA | 4/4 |

Lewis rats were irradiated (1,020 rad) with shielding of the right tibia. CsA-treated rats received 10 mg/kg/d for 18 d. (P < 0.005).

| Table III |
|----------|
| Adoptive Transfer of Syngeneic GVHD |
| Pretreatment of secondary recipient | Fraction of secondary recipients with GVHD |
| No radiation | 0/5 |
| 1,020 Rad | 12/13 |

10⁸ spleen cells from CsA-treated rats with syngeneic GVHD were transferred along with 6 × 10⁷ syngeneic Lewis marrow cells. Data pooled from four independent experiments (P < 0.005).

| Table IV |
|----------|
| Failure of Normal Spleen Cells to Prevent the Adoptive Transfer of Syngeneic GVHD |
| Cells transplanted | Fraction with clinical GVHD | Fraction with grade II acute GVHD | Onset day |
| GVHD spleen plus normal marrow | 3/3 | 3/3 | 12 |
| GVHD spleen plus normal marrow plus normal spleen | 3/3 | 3/3 | 12 |

Lethally irradiated Lewis rats were transplanted with 6 × 10⁷ Lewis marrow cells and 10⁴ Lewis spleen cells from rats with syngeneic GVHD. 10⁸ normal Lewis spleen cells were also given to some rats as indicated above.
Failure of Normal Spleen Cells to Prevent the Development of GVHD in CsA-treated Syngeneic Chimeras

| Cells transplanted | CsA mg/kg/d for 40 d | Fraction with clinical GVHD | Fraction with grade II histological GVHD |
|--------------------|----------------------|-----------------------------|-----------------------------------------|
| Marrow only        | 0                    | 0/6                         | 0/6                                     |
| Marrow and spleen  | 0                    | 0/5                         | 0/5                                     |
| Marrow only        | 7.5                  | 13/16                       | 15/16                                   |
| Marrow and spleen  | 7.5                  | 11/12                       | 12/12                                   |
| Marrow only        | 15                   | 11/16                       | 13/16                                   |
| Marrow and spleen  | 15                   | 3/3                         | 12/12                                   |

Lewis rats were lethally irradiated and treated with CsA or control diluent from day 0 to day 40. The rats were transplanted with $6 \times 10^7$ syngeneic Lewis marrow cells and $10^8$ spleen cells as indicated above.

also received an equal number of normal spleen cells. All rats developed grade II acute GVHD. As shown in Table V normal spleen cells also did not prevent the development of syngeneic GVHD when given at the time of marrow transplantation to CsA-treated syngeneic chimeras.

Discussion

The key observation presented in this paper is the consistent development of GVHD in CsA-treated rats after syngeneic or autologous bone marrow reconstituation, developing upon the discontinuation of CsA. Syngeneic GVHD may be adoptively transferred to irradiated but not normal syngeneic recipients. Syndromes similar to syngeneic and autologous GVHD have previously been described in neonatally thymectomized mice (10) and in neonatal mice given large doses of staphylococcal vaccines (11). In addition isolated cases of GVHD in patients receiving bone marrow from identical twins have been reported (12, 13). The existence of syngeneic and autologous GVHD provides compelling evidence that histocompatibility differences are not absolutely necessary for the development of GVHD.

CsA at low concentration in vitro reversibly inhibits the proliferative response of primary mixed lymphocyte reactions and prevents the development of cytotoxic T cells (14, 15). CsA appears to interfere with both the production and the acquisition of responsiveness to T cell growth factor (16–18). In vivo CsA induces a state of profound immunosuppression and is able to reversibly suppress allograft rejection and acute allogeneic GVHD (1–7). The mechanisms by which CsA causes syngeneic GVHD are at present enigmatic and the mechanisms by which normal rats resist the adoptive transfer to established GVHD remain to be identified.

Summary

Lethally irradiated rats treated with cyclosporin A (CsA) for 20–40 d develop classic graft-versus-host disease (GVHD) when reconstituted with syngeneic or autologous bone marrow, upon discontinuation of CsA, whereas normal rats do not. Syngeneic GVHD may be transferred to irradiated but not normal syngeneic
recipients. Normal spleen cells fail to prevent the development or adoptive transfer of syngeneic GVHD.

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