In Vivo Inhibition of Cytokine Responsiveness and Graft-Versus-Host Disease Mortality by Rapamycin Leads to a Clinical-Pathological Syndrome Discrete From That Observed With Cyclosporin A

By Bruce R. Blazar, Patricia A. Taylor, Angela Panoskaltsis-Mortari, Suren Sehgal, and Daniel A. Vallera

Rapamycin (RAPA) has been shown to be a highly effective means of reducing the lethality of graft-versus-host disease (GVHD) in B10.BR recipients of allogeneic C57BL/6 donor cells. RAPA-treated mice had no clinical (eg, weight loss, diarrhea, lethargy) or histologic evidence of classical acute or chronic GVHD but did develop a clinical-pathological syndrome consisting of ulcerative dermatitis, bile duct proliferation, and a nondestructive peribronchiolar pulmonary infiltration. Because RAPA was found to interfere with the development of self-reactive T cells, we wondered whether the RAPA-induced syndrome was related to failed negative selection or altered alloreactivity. We now show that the RAPA-induced syndrome is due to effects on mature, donor-derived alloreactive T cells. By titrating the number of T cells infused we were able to vary the syndrome incidence. In contrast to the syndrome seen after cyclosporin A (CsA) administration, the RAPA syndrome did not require an intact thymus and the disease could not be adoptively transferred. The addition of CsA (which blocks T-cell cytokine production) to RAPA (which blocks T-cell cytokine response) prevented the generation of this syndrome, suggesting that the tissue manifestations seen in RAPA only treated recipients were caused by cytokine production and release. RAPA also caused this alloimmune syndrome in recipients of minor histocompatibility antigen disparate donor cells, showing that the RAPA effects were not restricted to a single donor-recipient strain combination or to instances in which the donor and recipient were fully major histocompatibility complex disparate. We conclude that RAPA is a highly effective means of preventing murine acute GVHD, and that when combined with CsA, warrants consideration for human investigations.

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nic donor grafts and RAPA for 28 days. A proportion of the peripheral T cells with self-reactive specificities that escaped negative selection was shown to retain function as measured by in vitro proliferation to plate-bound anti-V/β monoclonal antibodies (MoAbs). Similar findings were noted for recipients of allogeneic donor BMS grafts. Because the proportion of T cells with potential antihost specificity (based on V/β phenotyping) was higher than observed in syngeneic BMT recipients, our two main hypotheses were: (1) the differential susceptibility to RAPA-induced disease in allogeneic versus syngeneic recipients might be due to the absolute number of mature T cells available in vivo to cause the autoimmune-like syndrome; or (2) RAPA treatment resulted in an unusual GVHD reaction.

To determine if the mechanism(s) involved with the RAPA syndrome compared to the well studied CsA-induced autoimmune syndrome were similar, we performed experiments in which recipients were thymectomized before BMT. Because an intact thymus is required for the CsA- but not RAPA-induced syndrome, these studies provided the first important information that these two syndromes were likely to be generated by different mechanisms. Rigorous T-cell depletion of the syngeneic donor inoculum has been shown to accentuate the CsA-induced autoaggression in primary recipients. Another strategy to accentuate the CsA syndrome involves the adoptive transfer of thymocytes or T cells from CsA-treated primary syngeneic BMT recipients to irradiated secondary syngeneic BMT recipients (eg, see ref 18). Similar experiments performed in the context of RAPA administration failed to cause any autoimmune-like manifestations, regardless of whether syngeneic or allogeneic BMT studies were performed. The failure to induce the RAPA syndrome occurred despite supplementing the RAPA-treated splenocytes with splenocytes from nontransplanted donor strain mice.

Additional experiments showed that the generation of the RAPA syndrome required both an ongoing allogeneic response and the continued administration of RAPA, and was not restricted to a single donor-recipient strain combination or to strain combinations differing at major histocompatibility complex (MHC) loci. The severity of the GVHD response (titrated by varying the number of donor splenocytes used to cause GVHD) was proportional to the incidence of the RAPA-induced syndrome, suggesting that RAPA reduced GVHD lethality but did not preclude the synthesis and release of cytokines capable of causing tissue damage. Because an ongoing allogeneic response is required, it is more appropriate to label this syndrome as alloimmune rather than autoimmune. Consistent with this hypothesis, the co-administration of RAPA, to inhibit cytokine responsiveness, and CsA, to inhibit cytokine production, prevents the RAPA-induced syndrome. Collectively, these studies suggest that the combined GVHD approach of RAPA + CsA warrants consideration for human GVHD preventive trials.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2b; Mls1c), B10.BR/SgSnJ (H-2b; Mls1c), C3H/HeJ (H-2d; Mls1c), CBA/J (H-2d; Mls1c), BALB/c (H-2d; Mls1c), and DBA/2 (H-2d; Mls1c), donor and recipient mice were purchased from Jackson Laboratory (Bar Harbor, ME) and the National Institutes of Health (Bethesda, MD). Female donor and recipient mice were 4 to 6 weeks and 6 to 8 weeks old, respectively, at the time of BMT. The mice were housed in a specific pathogen-free facility in microisolator cages.

BMT. Our transplant protocol has been described in detail. B10.BR or C57BL/6 recipients were conditioned with 8.0 or 9.0 Gy total body irradiation (TBI), respectively, administered from a Philips RT 250 Orthovoltage Therapy Unit (Philips Medical Systems, Brookfield, WI), filtered through 0.35-mm Cu at a final absorbed dose rate of 0.41 Gy/min at 225 kV and 17 mA. In one experiment in which the x-ray machine was unavailable, a Cesium was used to deliver a radiobiological equivalent dose of 9.5 Gy (B10.BR) or 10.5 Gy (C57BL/6) TBI. Donor BM was collected into RPMI 1640 medium by flushing it from the shafts of femurs and tibias. Twenty-five million BM and a total of 25 × 10^6 splenocytes as a source of GVHD-inducing T cells (termed BMS) were administered via caudal vein in 0.5 mL, vol, unless otherwise indicated. T-cell depletion (TCD) of donor inocula was performed by incubating BM (with or without supplemental splenocytes) with anti-Thy1.2 (antibody 30-H-12, rat IgG2b, provided by Dr David Sachs, Cambridge, MA) plus complement (C') (Pelfreez, Rogers, AK) as described previously. In some allogeneic experiments and in syngeneic experiments designed to attempt to adoptively transfer the autoimmune-like syndrome, anti-Thy1.2 was combined with anti-CD4 (antibody GK1.5, rat IgG2b, provided by Dr Frank Fitch, Chicago, IL) and anti-CD8 (2.43, rat IgGb, provided by Dr David Sachs) as a vigorous method of TCD. Recipients were monitored for survival, weight loss, and the onset of clinical (weight loss, alopecia, diarrhea, hunching, skin erythema) and histologic (see below) evidence of GVHD.

RAPA and CsA administration. RAPA was administered as a suspension in carboxymethylcellulose (CMC) (Sigma C5013, lot 109H361; Sigma, St Louis, MO). A stock solution of 2.5 mg/mL rapamycin in CMC was prepared. RAPA was resuspended in CMC at a final concentration of 0.2% CMC for injections and administered at doses of 1.5 mg/kg/dose intraperitoneally (ip) beginning on the day of or day before BMT and continuing daily for 14 days, and then three times per week until 1 month post-BMT. CsA was administered as a vehicle control. CsA, kindly provided by Dr P. Arcesse (Sandoz Pharmaceuticals, Hanover, NJ) was prepared from lyophilized material (Sandimmune, lot 3905092, concentration 50 mg/mL), resuspended in 0.2% CMC, and administered at doses of 10 mg/kg/dose according to the same schedule as described above for RAPA.

In vivo T-cell depletion and adult thymectomy (ATx). Anti-CD4 or anti-CD8 MoAb was administered at a dose of 100 μg on day 1 then weekly through day 27 post-BMT. The in vivo injection of anti-CD4 or anti-CD8 MoAb post-BMT is highly effective and has been shown to eliminate greater than 99% CD4+ or CD8+ T cells, respectively, for at least 2 weeks after discontinuation of the MoAbs as determined by flow cytometry (data not shown). Adult thymectomy was performed in some recipients before BMT by dissection under direct visualization. Histologic inspection of designated mice for remaining thymic remnants has confirmed the adequacy of this procedure for thymic removal. Because the mature postthymic T cells persist for a long period of time, thymectomy is combined with in vivo T-cell depletion.

Pathologic examination of tissues. Mice were killed and tissues were taken for histopathologic analysis. Samples were placed in either 10% neutral buffered formalin and then imbedded in paraffin or were immediately frozen. Tissues were sectioned, and stained with hematoxylin and eosin for histopathologic assessment. Samples were coded and read. Organs were scored positive for acute GVHD if there was single cell necrosis (skin, colon), crypt dropout (colon), peritropical infiltrate with acute necrosis (liver), or endothelialitis with a lymphocytic infiltrate (lung). These features are present only in mice with active acute GVHD, and not in normal mice or in irradiated recipients of syngeneic BMT. Tissues were also examined for
any evidence of chronic GVHD including inflammatory colon lesions, sclerodema, and pulmonary interstitial thickening as described in the literature. To determine if mice had the unique histologic manifestations of the RAPA syndrome, tissues were examined for signs of bile duct hyperplasia and pulmonary damage as previously described for RAPA-treated recipients of fully allogeneic donor grafts.

**Skin lesions.** Mice were scored for the onset of skin lesions post-BMT. These skin lesions had a distinctive characteristic appearance, occurring on the hindlimb and forelimb and were characterized by intense inflammation, scarring, and eventual contractures. In RAPA-treated mice, these lesions appeared either during or within 1 month after discontinuation of injections. Typically, the lesions progress over time. Mice that developed lesions did so within the first 2 months post-BMT and no new cases were noted even during observation periods as long as 202 days in RAPA-treated or control groups. Most (75%) of the mice that develop skin lesions had associated bile duct hyperplasia and no mice had bile duct proliferation without the associated skin lesions. Such skin lesions have never been observed in non- RAPA-treated, BMT mice in our laboratory, including circumstances in which mice with severe acute or chronic GVHD survived beyond the observation period used to score RAPA-treated mice or in allogeneic mice that have received other GVHD preventive therapies including CsA or FK506.

**Statistical analyses.** Groupwise comparisons of continuous data were made by Student’s t-test. Survival data were analyzed by life-table methods using the Mantel-Peto-Cox summary of chi square.

**RESULTS**

*In contrast to the CsA-induced autoimmune syndrome, an intact thymus is not required for the RAPA-induced syndrome.* We hypothesized that the RAPA-induced lesions seen in lethally irradiated recipients of fully allogeneic grafts may be due to a unique type of thymic dysplasia as a result of TBI and RAPA administration, as has been noted with the combined use of TBI and CSA. To determine if an intact thymus was required for the RAPA-induced syndrome, allogeneic BMT recipients were ATx 6 weeks before BMT. Skin ulcerations occurred in 8/24 (33%) RAPA-treated euthymic mice between days 24 and 50 post-BMT. Four of 28 (14%) of evaluable RAPA-treated ATx mice developed skin lesions (Table 1). Because the actuarial survival rates of ATx mice were significantly (P < .001) lower than euthymic mice similar to literature reports, there were fewer ATx mice that survived and could be observed for skin-lesion development. The true incidence of skin lesions in ATx and euthymic mice are likely to be fairly comparable when taking into account the proportion of ATx compared with euthymic mice that were evaluable for skin-lesion development during the critical time period post-BMT (typically 3 to 6 weeks) (Table 1). Thymectomy itself does not interfere with survival as thymectomized mice are observed for 6 weeks before BMT and no increased mortality rates have been observed in ATx mice that have received BMT in situations in which GVHD cannot occur due to the infusion of T-cell-depleted donor grafts. Regardless as to whether mice were euthymic or ATx, RAPA administration resulted in a significantly higher actuarial survival rate compared with their respective controls (P = .000001: .00014). Thus, an intact thymus is not a prerequisite for the generation of this syndrome.

*In contrast to the CsA syndrome, the RAPA-induced syndrome cannot be adoptively transferred from RAPA-treated syngeneic or allogeneic primary recipients.* Because CsA-induced autoaggression is more severe in lethally irradiated secondary recipients that receive syngeneic thymocytes from mice given lethal irradiation and CsA, we asked if the classical RAPA-induced syndrome, not previously observed in primary recipients, could be induced in secondary recipients of RAPA-treated thymocytes or splenocytes. B10.BR mice were chosen as recipients because RAPA has been previously shown to inhibit the clonal deletion of Vβ5 and Vβ11 T cells (typically negatively selected in the B10.BR thymus). These T cells are functional. Irradiated B10.BR recipients of B10.BR BM, rigorously depleted of T cells (anti-Thy 1.2 + anti-CD4 + anti-CD8 + C' treated), were treated with RAPA or CMC (n = 12/group). Nine days after RAPA or CMC discontinuation (on day 38 post-BMT), 33 x 10^6 splenocytes (containing CD4+ cells [3%, 10%] and CD8+ cells [2%, 3%], respectively) or 12 x 10^6 thymocytes were transferred along with B10.BR TCD BM from nontransplanted donors into lethally irradiated secondary B10.BR recipients (n = 6/group). None of the secondary recipients were noted to have skin lesions, pulmonary infiltrates, or bile duct proliferation during the 81-day observation period, despite the fact that CsA-induced autoaggression can be adoptively transferred with 5 x 10^6 splenocytes to irradiated mouse recipients (Table 2). The RAPA-induced syndrome is not able to be adoptively transferred under the conditions used for these studies.

Because an even higher proportion of T cells with anti-host-reactive specificities were observed in RAPA-treated allografted mice compared with syngeneic recipients and
because only allografted mice were susceptible to the disease syndrome, we asked whether the disease process could be adoptively transferred into secondary recipients by infusing allogeneic cells. Donor anti-host-reactive T cells from RAPA-treated transplanted mice might be capable of causing the autoimmune-like lesions in susceptible secondary recipients. Although the incidence of skin ulcers was 50% in RAPA-treated primary recipients, the disease was not transferable (Table 2). Allogeneic T cells from RAPA-treated primary recipients were not capable of generating the disease in secondary recipients at the cell doses used.

One explanation for the failure to transfer the RAPA syndrome is the possibility that GVHD capacity of the splenocytes which were used for adoptive transfer was impaired by RAPA treatment. For example, if donor antihost counterregulatory cells were generated by RAPA, the adoptive transfer of syngeneic or allogeneic splenocytes into secondary recipients would fail to transfer the disease. To address this possibility, splenocytes from RAPA-treated recipients were mixed with splenocytes from nontransplanted controls to determine if RAPA treatment prevented GVHD generation of naïve allogeneic splenocytes.

Splenocytes from RAPA- or CMC-treated C57BL/6 → B10.BR chimeras were mixed 1:1 with C57BL/6 splenocytes from nontransplanted C57BL/6 controls. A total of $25 \times 10^6$ cells were given to irradiated B10.BR secondary recipients (Table 3). Zero to 13% of secondary recipients survived, regardless of whether the BMS inocula contained splenocytes from RAPA- or CMC-treated chimeras. Survival was high (100%) in secondary recipients of splenocytes from primary B10.BR recipients that received C57BL/6 TCD BMS since these cells would be expected to be tolerant of host (B10.BR) alloantigens. Similar to the data obtained with splenocytes from RAPA-treated allogeneic chimeras, 0% of B10.BR secondary recipients of splenocytes from RAPA-treated C57BL/6 → C57BL/6 chimeras survived when the donor inocula contained splenocytes from nontransplanted control C57BL/6 donors. The failure to adoptively transfer the RAPA syndrome in secondary recipients of syngeneic or allogeneic BMS was not because of the generation of counterregulatory cells in RAPA-treated mice which inhibited anti-host responses in vivo.

The intensity of GVHD determines whether the RAPA-induced syndrome will be observed. To determine whether allogeneic donor T cells were required for the generation of skin ulcerations, irradiated B10.BR recipients were transplanted with TCD or non-TCD C57BL/6 BMS and then treated with RAPA. By 3 weeks post-BMT, skin ulcerations developed in 8 of 12 (67%) RAPA recipients of non-TCD BMS (Table 4). None of the RAPA recipients of TCD BMS had skin lesions. These data showed that allogeneic T cells were required for RAPA-induced skin ulcerations.

To determine whether donor CD4+ and/or CD8+ T cells were required for the syndrome, anti-CD4 and/or anti-CD8 MoAbs were given for 1 month post-BMT to irradiated, RAPA-treated allogeneic recipients. Depletion of CD4+ but not CD8+ T cells prevented the development of the skin lesions in each of the 12 mice depleted of CD4+ T cells in vivo and given RAPA treatment. Because CD4+ T cells have a more pronounced effect than CD8+ T cells on GVHD-induced mortality in this strain combination, we could not conclude whether the CD4+ T cells were directly responsible for causing the skin lesions or were more potent in modifying the intensity of the GVHD reaction than CD8+ T cells.

To determine if the intensity of the GVHD reaction correlated with the development of skin lesions, the numbers of
Table 3. RAPA-Treated Splenocytes Do Not Inhibit GVHD Generation by Naive Allogeneic BMS

<table>
<thead>
<tr>
<th>Donor Type</th>
<th>% Donor T Cells</th>
<th>Host</th>
<th>% Survival (day post-BMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allogeneic chimeras as donors for secondary BMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6-B10.R (TCD BMS, CMC)</td>
<td>16</td>
<td>B10.BR</td>
<td>100 (d57)</td>
</tr>
<tr>
<td>C57BL/6-B10.R (TCD BMS, CMC) + C57BL/6 (BMS)</td>
<td>22</td>
<td>B10.BR</td>
<td>13 (d57)</td>
</tr>
<tr>
<td>C57BL/6-B10.R (BMS, RAPA)</td>
<td>3</td>
<td>B10.BR</td>
<td>84 (d57)</td>
</tr>
<tr>
<td>C57BL/6-B10.R (BMS, RAPA) + C57BL/6 (BMS)</td>
<td>15</td>
<td>B10.BR</td>
<td>0 (d27)</td>
</tr>
<tr>
<td>Syngeneic chimeras as donors for secondary BMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6-C57BL/6 (BMS, CMC)</td>
<td>19</td>
<td>B10.BR</td>
<td>0 (d30)</td>
</tr>
<tr>
<td>C57BL/6-C57BL/6 (BMS, CMC) + C57BL/6 (BMS)</td>
<td>25</td>
<td>B10.BR</td>
<td>0 (d28)</td>
</tr>
<tr>
<td>C57BL/6-C57BL/6 (BMS, RAPA)</td>
<td>11</td>
<td>B10.BR</td>
<td>25 (d57)</td>
</tr>
<tr>
<td>C57BL/6-C57BL/6 (BMS, RAPA) + C57BL/6 (BMS)</td>
<td>15</td>
<td>B10.BR</td>
<td>0 (d66)</td>
</tr>
<tr>
<td>Nontransplanted mice as donors for BMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6 (BMS)</td>
<td>26</td>
<td>B10.BR</td>
<td>0 (d28)</td>
</tr>
</tbody>
</table>

Primary recipients (n = 8-12/group) were lethally irradiated, transplanted with TCD BM and either TCD splenocytes [designated TCD BMS in parentheses] or non-TCD splenocytes [designated BMS in Table (25 x 10^6) cells from allogeneic or syngeneic C57BL/6 donors, and given CMC or RAPA for 1 month post-BMT as indicated in Table 5. Secondary recipients (n = 8/group) were irradiated and transplanted with TCD BM and a total of 25 x 10^6 splenocytes obtained from nontransplanted C57BL/6 controls, chimeras, or a 1:1 mixture of both. The total T-cell content was determined by flow cytometry and derived by adding the percentage of CD4+ or CD8+ T cells together. The CD4:CD8 ratio in all instances was determined by flow cytometry and derived by adding the percentage of CD4+ or CD8+ T cells together. The CD4:CD8 ratio in all instances was greater than 1 (data not shown). The actuarial survival rates of the secondary recipients are shown.

Table 4. In Vitro Removal of Allogeneic T Cells Prevents RAPA-Induced Skin Ulcerations

<table>
<thead>
<tr>
<th>Donor Type</th>
<th>Cell Type</th>
<th>Host</th>
<th>% Survival (day post-BMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6-B10.R (TCD BMS, CMC)</td>
<td>TCD BMS</td>
<td>B10.BR</td>
<td>CMC</td>
</tr>
<tr>
<td>C57BL/6-B10.R (TCD BMS, CMC)</td>
<td>TCD BMS</td>
<td>B10.BR</td>
<td>RAPA</td>
</tr>
<tr>
<td>C57BL/6-B10.R (TCD BMS, CMC)</td>
<td>Non-TCD BMS</td>
<td>B10.BR</td>
<td>CMC</td>
</tr>
<tr>
<td>C57BL/6-B10.R (TCD BMS, CMC)</td>
<td>Non-TCD BMS</td>
<td>B10.BR</td>
<td>RAPA</td>
</tr>
</tbody>
</table>

Nontransplanted recipients were lethally irradiated and transplanted with C57BL/6 TCD BM and either TCD C57BL/6 splenocytes or non-TCD C57BL/6 splenocytes (25 x 10^6 cells). Groups receiving non-TCD BMS were randomized to receive CMC or RAPA (1.5 mg/kg/dose) for 1 month post-BMT. Skin lesions were recorded throughout the observation period.
Table 5. The Incidence of RAPA-Induced Skin Ulcerations Is Higher in B10.BR Recipients of Larger Numbers of C57BL/6 Donor Splenocytes

<table>
<thead>
<tr>
<th>Donor</th>
<th>Spleen No. (× 10⁷)</th>
<th>Host</th>
<th>RAPA and/or MoAb</th>
<th>Skin Lesions (%)</th>
<th>% Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>25</td>
<td>B10.BR</td>
<td>RAPA</td>
<td>5/12 (43)</td>
<td>92</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>25</td>
<td>B10.BR</td>
<td>RAPA + anti-CD8</td>
<td>2/8 (25)</td>
<td>75</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>15</td>
<td>B10.BR</td>
<td>RAPA</td>
<td>1/12 (0)</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5</td>
<td>B10.BR</td>
<td>RAPA</td>
<td>0/12 (0)</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1</td>
<td>B10.BR</td>
<td>RAPA</td>
<td>0/12 (0)</td>
<td>92</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1</td>
<td>B10.BR</td>
<td>RAPA + anti-CD8</td>
<td>0/8 (0)</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>25</td>
<td>B10.BR</td>
<td>CMC</td>
<td>0/12 (0)</td>
<td>12</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>25</td>
<td>B10.BR</td>
<td>CMC + anti-CD8</td>
<td>0/8 (0)</td>
<td>12</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>15</td>
<td>B10.BR</td>
<td>CMC</td>
<td>0/12 (0)</td>
<td>25</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5</td>
<td>B10.BR</td>
<td>CMC</td>
<td>0/12 (0)</td>
<td>87</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1</td>
<td>B10.BR</td>
<td>CMC</td>
<td>0/12 (0)</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1</td>
<td>B10.BR</td>
<td>CMC + anti-CD8</td>
<td>0/8 (0)</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>25 (TCD)</td>
<td>B10.BR</td>
<td>None</td>
<td>0/8 (0)</td>
<td>100</td>
</tr>
</tbody>
</table>

B10.BR recipients were irradiated, transplanted with C57BL/6 TCD BM and C57BL/6 non-TCD or TCD splenocytes at the indicated cell numbers, and given CMC or RAPA (1.5 mg/kg/dose) for 1 month post-BMT. Some mice also received anti-CD8 MoAb weekly from day -1 to + 29 post-BMT. Skin lesions were recorded throughout the observation period. Ninety-day actuarial survival rates were calculated and are listed.

B10.BR → CBA/J strain combination, RAPA administration was partially effective \( (P = 0.00045 < \text{CMC}; P = 0.0021 < \text{TCD}) \) in preventing GVHD-induced lethality. Skin lesions, consisting of ulcerations of both hindlimbs and the perianal area, were observed in 5/18 (28%) of recipients and some but not all of the RAPA-treated mice were GVHD-free. In the B10.D2 → DBA/2 strain combination, RAPA-treated mice also had significantly higher actuarial survival rates (37%) than the CMC group (0%) but lower than the TCD group (89%) \( (P = 0.007 < \text{CMC}; P = 0.015 < \text{TCD}) \). Despite

Table 6. The Incidence of RAPA-Induced Skin Ulcerations in Other Strain Combinations Differing at MHC Class I + II or Minor Histocompatibility Disparities Alone

<table>
<thead>
<tr>
<th>Donor</th>
<th>Spleen No. (× 10⁷)</th>
<th>Host</th>
<th>RAPA and/or MoAb</th>
<th>Skin Lesions (%)</th>
<th>% Survival Rate (day post-BMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.D2</td>
<td>25</td>
<td>C57BL/6</td>
<td>RAPA</td>
<td>0/6 (0)</td>
<td>80 (d56)</td>
</tr>
<tr>
<td>B10.D2</td>
<td>50</td>
<td>C57BL/6</td>
<td>None</td>
<td>0/6 (0)</td>
<td>0 (d34)</td>
</tr>
<tr>
<td>B10.D2</td>
<td>25</td>
<td>C57BL/6</td>
<td>None</td>
<td>0/6 (0)</td>
<td>0 (d33)</td>
</tr>
<tr>
<td>B10.D2</td>
<td>15</td>
<td>C57BL/6</td>
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<td>0/6 (0)</td>
<td>0 (d32)</td>
</tr>
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<td>15</td>
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<td>RAPA</td>
<td>0/10 (0)</td>
<td>50 (d100)</td>
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<td>0 (d8)</td>
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<td>0 (d42)</td>
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<td>25</td>
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<td>RAPA</td>
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<td>0/8 (0)</td>
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<td>C57BL/6</td>
<td>None</td>
<td>0/8 (0)</td>
<td>63 (d60)</td>
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Lethally irradiated recipients were transplanted with TCD or non-TCD donor BM. Recipients were randomized to receive CMC, RAPA (1.5 mg/kg/dose), or no injections. The incidence of skin lesions was recorded throughout the observation period. Actuarial survival rates were calculated and are listed along with the last day of observation in parentheses.
the more aggressive nature of the GVHD response in the B10.D2 → DBA/2 compared with the B10.BR → CBA/J strain combination (100% lethality in the CMC group by day 24 and 72 post-BMT, respectively), none of the RAPA-treated mice developed skin lesions. The mean weight curves in both strain combinations were consistent with the actuarial survival data. Thus, in a rapidly lethal, CD8+ T-cell–dependent GVHD system caused by miH antigens only disparities, RAPA administration can be effective in reducing GVHD without inducing the characteristic skin manifestations of the RAPA syndrome. These data indicate that variables other than GVHD intensity or dependency on either CD4+ or CD8+ T cells can contribute to the RAPA syndrome.

The coadministration of CsA along with RAPA prevents the development of the RAPA-induced skin lesions. The data shown above suggest that RAPA modifies the GVHD reaction such that T cells are impaired in their GVHD effects but are capable of causing tissue injury. Because RAPA interferes with T-cell cytokine responses but not cytokine production, it is possible that T-cell elaborated cytokines were continuing to be produced and were responsible for certain tissue changes such as bile duct proliferation. Therefore, CsA (10 mg/kg/dose) was added to RAPA to inhibit cytokine production and/or modify the GVHD response in the C57BL/6 → B10.BR model system (Table 7). The addition of CsA to RAPA eliminated the skin lesions associated with RAPA treatment. However, bile duct proliferation was seen in the majority of RAPA + CsA treated recipients. Actuarial survival was reduced and the mean weight curves showed that recipients of CsA + RAPA had mean weights that were approximately 20% lower than RAPA-treated recipients throughout the post-BMT period. Because mice treated with CsA + RAPA did have histologic evidence of GVHD at the time of death, it is not clear whether the lower mean weights in the CsA + RAPA-treated group were caused by toxicity or an adverse effect of CsA, when given along with RAPA, on GVHD prevention. Nonetheless, CsA was able to inhibit the generation of RAPA-induced skin lesions without increasing actuarial survival rates or improving the clinical appearance of murine recipients of fully allogeneic BMS. Because bile duct proliferation was still present in the absence of skin lesions in RAPA + CsA-treated recipients, either the pathophysiological mechanism(s) involved in these processes are separable or the skin lesions are most sensitive to CsA inhibition at the CsA dose tested.

## DISCUSSION

The major findings of this study are (1) RAPA administration, in strain combinations differing at MHC or miH loci, causes a unique spectrum of tissue injury, distinct from classical acute or chronic GVHD and distinct mechanistically as well as pathologically distinct from the CsA-induced autoimmune syndrome; and (2) CsA added to RAPA prevents the development of these RAPA-induced tissue manifestations. Therefore, this syndrome is more appropriately called an alloimmune syndrome.

Could the RAPA tissue manifestations be a result of acute, subacute, or chronic GVHD? Our data argue that the RAPA is distinct from classical GVHD tissue injury. RAPA-treated mice remain clinically healthy during the entire observation period of greater than 200 days. Histological findings are distinguishable from acute or chronic GVHD. Even though some of the non–RAPA-treated recipients of splenocytes survived long-term, these animals showed evidence of GVHD and no evidence of the RAPA syndrome. The RAPA syndrome occurs as early as 3 to 4 weeks post-BMT, a time when some of the non–RAPA-treated controls are still alive and the RAPA-treated mice are continuing to receive RAPA. Thus, this syndrome is not simply a result of animals with GVHD surviving sufficiently long to have these tissue lesions be analyzed.

What are the possible mechanisms involved in the RAPA-induced syndrome? The most likely explanation for the RAPA effects are that donor T cells are impaired in their cytokine response, which relates to their impaired GVHD capacity, but are intact in terms of cytokine production, which relates to the tissue injury. Although RAPA may be effective in terms of preventing GVHD lethality, cytokines released during the typical GVHD generation process, which would not be directly affected by RAPA, may result in a GVH-induced tissue injury without lethality. Thus, RAPA administration may alter the GVH reaction that leads to a modified form of GVHD. For example, T-cell–derived IL-6 may be responsible for the bile duct proliferative process. In this scenario, donor T cells encountering host peptides/antigens typically presented in the context of MHC molecules would become activated and release IL-6, TCR activation, while CsA inhibitable, would still occur with RAPA treatment. Sufficient IL-6 would be released such that bile duct proliferation (and possibly skin ulcerations) would oc-
cur but lethal GVHD would not result because donor T cells are not expanded to a critical threshold due to poor cytokine responsiveness and amplification. Alternatively, RAPA administration might cause a shift in the functional properties of donor T cells such that the critical T-cell phenotype is preferentially impaired. In this scenario, Th1 cells may be more susceptible to RAPA than Th2 cells. Support for the hypothesis that T-cell-mediated cytokine release is necessary for the tissue injury is the fact that the incorporation of CsA to inhibit T-cell-mediated cytokine production, along with RAPA, eliminates the RAPA-induced syndrome even though the animal health and GVHD, per se, has not been favorably impacted by CsA. Because some mice had skin lesions without bile duct proliferation and CsA co-administration with RAPA prevented the skin lesions without affecting bile duct proliferation, there may be different mechanisms involved in these two processes even though these may be interrelated. The likelihood of observing the RAPA syndrome would then be dependent both on the particular pathophysiology of GVHD in a given strain combination and the conditions used for GVHD generation (see Table 6).

The susceptibility of CD4+ and CD8+ T cells to RAPA inhibition and the relative contribution of each of these T-cell subpopulations which vary depending upon strain combination might indicate whether the result will be GVHD inhibition, RAPA-associated tissue injury, or both. Because CD4+ T cells are more critical than CD8+ T cells in initiating the GVHD response in most donor-recipient pairs differing at MHC loci, one could speculate that CD4+ but not CD8+ T cells are preferentially inhibited by RAPA so that GVHD is markedly reduced but the CD8+ T cells remain intact. The CD8+ T cells that remain could then mediate the tissue injury in certain susceptible strain combinations. Several lines suggest against this hypothesis. RAPA was effective in reducing GVHD-induced mortality in strain combinations differing only at miH antigens, which are predominantlymediated by CD8+ but not CD4+ donor T cells. CD8+ T-cell elimination in B10.BR recipients of C57BL/6 BMS did not eliminate the RAPA-inhibited syndrome. In other experiments involving GVHD induced by highly purified CD8+ T cells recognizing an MHC class I only disparity, RAPA treatment was highly effective in preventing GVHD-induced lethality (Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Sehgal SN, Vallera DA: unpublished data, September 1995). Therefore, we favor the explanation that the RAPA syndrome is dependent on the pathophysiology of GVHD in a given strain combination, and not whether CD4+ versus CD8+ T cells were more important to generating GVHD.

In summary, the current studies have shown that RAPA is effective in preventing GVHD-induced lethality in donor-recipient strain combinations differing at MHC class I or II loci or miH loci. In some of these GVHD systems, recipients are susceptible to the development of skin ulcerations and a histological process typified by bile duct proliferation and pulmonary peribronchial infiltrate not characteristic of but perhaps related to classical GVHD. In contrast to CsA-induced autoimmunity, the RAPA syndrome is thymus-independent, requires mature donor T cells, has only been observed in allogeneic BMT recipients, and cannot be adoptively transferred. Because the addition of CsA to RAPA prevents the alloimmune disease independently of any perceived benefit on GVHD, the most likely explanation is that RAPA inhibits the cytokine responsiveness required for GVHD-induced lethality but not the cytokine production required for the alloimmune syndrome. Future studies will be needed to determine more precisely what mechanism(s) are responsible for the development of the alloimmune complications in some but not other strains of mice. The data in the present study have implications for the use of RAPA in clinical BMT and further suggest that the combined use of CsA and RAPA might avert the occurrence of this alloimmune reaction in susceptible humans.

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BR Blazar, PA Taylor, A Panoskaltsis-Mortari, S Sehgal and DA Vallera

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