The immunosuppressant Rapamycin Blocks In Vitro Responses to Hematopoietic Cytokines and Inhibits Recovering But Not Steady-State Hematopoiesis In Vivo

By Valerie F.J. Quesniaux, Susi Wehrli, Carolina Steiner, Joanne Joergensen, Henk-Jan Schuurman, Peter Herrmann, Max H. Schreier, and Walter Schuler

The immunosuppressive drug rapamycin suppresses T-cell activation by impairing the T-cell response to lymphokines such as interleukin-2 (IL-2) and interleukin-4 (IL-4). In addition, rapamycin blocks the proliferative response of cell lines to a variety of hematopoietic growth factors, including interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), and kit ligand (KL), suggesting that it should be a strong inhibitor of hematopoiesis. In this report, we studied the effects of rapamycin on different hematopoietic cell populations in vitro and in vivo. In vitro, rapamycin inhibited the proliferation of primary bone marrow cells induced by IL-3, GM-CSF, KL, or a complex mixture of factors present in cell-conditioned media. Rapamycin also inhibited the multiplication of colony-forming cells in suspension cultures containing IL-3 plus interleukin-1 (IL-1) or interleukin-11 (IL-11) plus KL. In vivo, treatment for 10 to 28 days with high doses of rapamycin (50 mg/kg/d, orally) had no effect on myelopoiesis in normal mice, as measured by bone marrow cellularity, proliferative capacity, and number of colony-forming progenitors. In contrast, the same treatment strongly suppressed the hematopoietic recovery normally seen 10 days after an injection of 5-fluorouracil (5-FU; 150 mg/kg, intravenously [iV]). Thus, rapamycin may be detrimental in myelocompromised individuals. In addition, the results suggest that the rapamycin-sensitive cytokine-driven pathways are essential for hematopoietic recovery after myelodepression, but not for steady-state hematopoiesis.

© 1994 by The American Society of Hematology.

Rapamycin is an immunosuppressive drug that inhibits T-cell activation by interfering with signals induced by the T-cell growth factors IL-2 or IL-4. However, the effect of rapamycin is not limited to the T-cell lineage. It also inhibits the response of endothelial cells and fibroblasts to fibroblast growth factor-beta (FGF-beta) and the response of hematoma cells to insulin, as well as that of mast cells and basophilic cell lines to IL-3, but is not a general cytotoxic. We show here that, in vivo, rapamycin effectively blocks the proliferative responses of various cell lines, primary bone marrow cells, and bone marrow progenitors to all the hematopoietic cytokines tested, namely the ligand for c-kit (KL), G-CSF, GM-CSF, interleukin-1 (IL-1), IL-3, interleukin-6 (IL-6), and interleukin-11 (IL-11). This suggested that rapamycin is a general inhibitor of cytokine-driven proliferation. Rapamycin thus appears to be an interesting tool to address the role of cytokines in hematopoiesis.

In the present study, we report on different in vivo effects of rapamycin administration on steady-state and recovering hematopoiesis.

MATERIALS AND METHODS

Growth factors and drugs. The following recombinant human (h) and murine (m) growth factors were used: hIL-1β, hIL-3, hGM-CSF, mGM-CSF, and mIL-3 (Sandoz Pharma, Basel, Switzerland), hIL-11 (Genetics Institute, Cambridge, MA), mKL (kind gift of Dr

From Preclinical Research, Sandoz Pharma Ltd, Basel, Switzerland.

Submitted December 13, 1993; accepted May 9, 1994.

Address reprint requests to Valerie F.J. Quesniaux, PhD, Preclinical Research 386/155, Sandoz Pharma Ltd, CH-4002 Basel, Switzerland.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.
the presence of mIL-3, mGM-CSF, mKL, together with increasing concentrations of drug. Cell proliferation of stimulation of proliferation were selected. After cells were seeded at cytokines were titrated, and concentrations within the linear range 3 days in culture by \(^{3}H\) thymidine incorporation over 5 hours. The results are the mean from WEHI cells and incubated in 10% fetal calf serum (FCS) containing RPMI in bone marrow cells.

S. Gillis, Immunex, Seattle, WA. Cyclosporine (CsA, 0.83 mmol/L; Sandimmune) FK-506 (2 mmol/L), and rapamycin (2 mmol/L) were from Sandoz Pharma Ltd. They were dissolved in absolute ethanol.

Cytokine bioassays. The M07-E cell line was used as previously described\(^{11}\) to study the proliferative response to hIL-3, hGM-CSF, and mKL. Briefly, cells were washed twice and incubated in microtiter plates (2 \(\times\) 10\(^{4}\) cells/well) for 3 days in the presence of cytokines together with increasing concentrations of drug. Cell proliferation was measured after 5 hours of \(^{3}H\) thymidine incorporation.

Bone marrow cell proliferation. Femoral murine bone marrow cells were seeded at 5 to 10 \(\times\) 10\(^{4}\) cells/0.2 mL in 96-well plates and incubated in 10% fetal calf serum (FCS) containing RPMI in the presence of mIL-3, mGM-CSF, mKL, or conditioned medium from WEHI cells (4%) and L929 cells (1.5%) as indicated. All cytokines were titrated, and concentrations within the linear range of stimulation of proliferation were selected. After 2 to 4 days of incubation at 37°C, cell proliferation was measured by \(^{3}H\) thymidine incorporation over 5 hours. Cell suspensions from individual mice were tested in triplicate.

Bone marrow cell colony assay. Methyl cellulose culture of murine bone marrow cells (5 \(\times\) 10\(^{4}\) cells/mL) were prepared in Iscove's modified Dulbecco's medium (IMDM) containing 4% FCS, bovine serum albumin (BSA) fraction V, transferrin, and lipids according to Iscove et al\(^{12}\) in the presence of erythropoietin (Epo, 0.08 U/mL) plus mIL-3 (30 ng/mL) and hIL-11 (50 ng/mL). Bone marrow cells from in vivo-treated mice were pooled per group. Cells from 5-fluorouracil (5-FU)--treated mice were titrated (0.1 to 10 \(\times\) 10\(^{4}\) cells/mL) to ensure comparable numbers of colonies per culture. Macrophs (\(>\)1 mm) multilineage colonies comprising erythroid, megakaryocytes, and other myeloid cells, erythroid colonies (either pure or mixed with megakaryocytes), and GM colonies were counted on day 9. The colonies were identified by their morphology, and were periodically picked, spread, and stained (Wright-Giemsa) for confirmation. Results from four to six plates are pooled for each determination.

Bone marrow cell suspension cultures. Suspension cultures consisted of mouse bone marrow cells (10\(^{5}\) cells/mL) cultured in 1 mL IMDM containing 5% FCS, \(\alpha\)-thioglycerol, and 0.1% BSA in 35-mm plastic bacteriologic petri plates, according to Iscove et al\(^{12}\) Human IL-1\(\beta\) (10 ng/mL) plus mIL-3 (30 ng/mL) or hIL-11 (50 ng/mL) plus mKL (100 ng/mL) were added to the cultures as indicated. After 4 days of incubation at 37°C, the cells were gently resuspended and harvested. The content of colony-forming cells was determined in secondary methylcellulose cultures containing optimized concentrations of mIL-3 (30 ng/mL), hIL-1\(\beta\) (10 ng/mL), and Epo (0.08 U/mL). Bone marrow cells from 5-FU--treated mice were titrated from 0.1 to 10 \(\times\) 10\(^{4}\) cells/mL in suspension culture. Duplicate suspension cultures were assayed in four methylcellulose cultures.

In vivo experiments. Female mice (8 to 9 weeks old C57B1/6,
RAPAMYCIN INHIBITS RECOVERING HEMATOPOIESIS

**Figure 1.** Rapamycin is a potent inhibitor of cell line proliferation driven by hematopoietic cytokines. Figure 1 shows that the proliferative response of M07-E cells to hIL-3, hGM-CSF, or mKL was inhibited by rapamycin, but was not affected by two other immunosuppressive drugs, CsA and the structural analog of rapamycin, FK-506.23 Rapamycin also inhibited the proliferation of other cell lines such as B13-29, FDCP-9, and 32D stimulated with hIL-6, hG-CSF, or mIL-3, respectively (data not shown). In all cases, the dose of rapamycin resulting in half-maximum inhibition was in the subnanomolar range (Fig 1), similar to what was previously reported for the IL-2-driven proliferation of T cells.13 Rapamycin was not cytotoxic at these concentrations because its effect could be completely antagonized by a 100-fold molar excess of FK-506, similar to what was previously reported for T cells.24 Thus, in vitro, rapamycin effectively blocked the proliferative response of cell lines to all hematopoietic cytokines tested.

**Figure 2.** Effect of rapamycin on primary bone marrow cell proliferation. Rapamycin (e), but not CsA (A) or FK-506 (C), inhibits the proliferation of murine bone marrow cells in response to mIL-3 (A), mGM-CSF (B), mKL (C), or to a combination of WEHI and L929 cell-conditioned media (D). The results are the mean ± SEM of two independent experiments. Proliferation in the absence of inhibitor was 34,610 ± 1,790 cpm/10^5 cells stimulated with IL-3 (100 ng/mL), 74,901 ± 2,215 cpm/10^5 cells stimulated with GM-CSF (10 ng/mL), 24,392 ± 4,095 cpm/10^5 cells stimulated with KL (150 ng/mL), and 37,999 ± 2,730 cpm/10^5 cells stimulated with WEHI plus L929 cell-conditioned media (7.5% and 3%, respectively).

**RESULTS**

Rapamycin inhibits the proliferation of primary bone marrow cells in response to cytokines. As shown in Figure 2, the proliferation of primary murine bone marrow cells incubated...
from two independent experiments and are expressed as the ratio of concentration cultures. Bone marrow cells (10^5 CBA/J cells/mL) were incubated for 4 days in the presence of mIL-3 (30 ng/mL) plus mIL-1β (10 ng/mL) with increasing concentrations of rapamycin added on day 0. The cultures were supplemented with 90% of the initial rapamycin amount on day 1, 2, and 3. On day 4, the cells were harvested and the content of multilineage (W), erythroid (B), and GM progenitors was tested in secondary methylcellulose cultures. The results are pooled between the number of colonies recovered after suspension culture, and the number of colonies obtained from the original bone marrow cell population (10 multilineage, 28 erythroid, and 80 GM colonies per 10^6 cells). The dose-dependent inhibition of CFC multiplication by rapamycin was statistically significant (regression analysis using log-transformed rapamycin concentrations, P < .01).

for 2 days with either mIL-3, mGM-CSF, mKL, or with a combination of WEHI and L929 cell conditioned media as a source of cytokines, was inhibited up to 70% by nanomolar concentrations of rapamycin (half-maximum inhibition, ~0.3 nmol/L). The two other immunosuppressive drugs, CsA and FK-506, were inactive (Fig 2). FK-506 completely antagonized the inhibitory effect of rapamycin on bone marrow cell proliferation at a 100-fold molar excess (not shown). Thus, rapamycin also blocked the proliferative response of primary bone marrow cells to cytokines in vitro.

Rapamycin inhibits the multiplication of hematopoietic progenitors in vitro. The proliferation and differentiation of murine hematopoietic progenitors is classically studied in colony assays where bone marrow cells are incubated for 7 to 9 days in semisolid culture. However, rapamycin is unstable in culture medium incubated at 37°C, its activity decreasing by 10-fold per 24 hours of incubation (Quesniaux VFJ, Wehrli S, Wioland C, Schuler W, Schreier MH: submitted) and, therefore, cannot be tested in 7 to 9 days semisolid cultures. We thus studied the effect of rapamycin on hematopoietic progenitors in a two-step culture assay where precolonony forming cells (pre-CFC) were first allowed to expand during 4 days in liquid culture in the presence of either IL-3 plus IL-1β or IL-11 plus KL. Cultures containing rapamycin were supplemented with 90% of the initial amount of rapamycin after 1, 2, and 3 days to compensate for rapamycin instability. The number of CFC recovered after 4 days in culture, as well as the input number of CFC in the initial bone marrow cell population, were assessed in secondary colony assays. Figure 3 shows that the multiplication of erythroid, myeloid, and multilineage CFC progenitors in response to IL-3 plus IL-1 was dose-dependently inhibited by rapamycin. Similar results were observed in bone marrow suspension culture stimulated with IL-11 plus KL (data not shown).

Rapamycin is not myelotoxic for normal mice. The effect of rapamycin on normal hematopoiesis was then studied in C57Bl/6 mice treated for 10 days with 25 or 100 mg/kg rapamycin orally daily. These rapamycin treatments were immunosuppressive, as they prevented the rejection of allogeneic vascularized heart transplants in the same strain of mice (see Table 3 for dose response). There were no signs of toxicity, such as body weight loss. In two independent experiments, the bone marrow cellularity of normal mice treated for 10 days with rapamycin was similar to that of untreated animals (Table 1). In histologic sections, the density and cytology of the bone marrow cell populations appeared normal. The absolute number of bone marrow myeloid progenitors, ie, erythroid- and GM-CFC was slightly increased after rapamycin treatment at both doses (also seen in the four experiments depicted in Table 2 and Fig 4). Although the number of multilineage CFC appeared to be decreased after rapamycin treatment in these animals, this was not consistently observed in four other experiments (1,038 multi-CFC per femur after rapamycin treatment v 528 in control in experiment 1, Table 2; 1,092 v 844 in experiment 2, Table 2; 2,389 v 3,313 and 1,604 v 1,186 in the two experiments shown in Fig 4). The ability of bone marrow cells to proliferate in response to cytokines ex vivo was essentially unchanged (Table 1). The spleen and thymus weights were significantly reduced with both doses of rapamycin (Table 1). Histologically, thymic cortical atrophy and some atrophy of the follicles in spleen and the lymph nodes were evident. Although bone marrow myeloid cells seemed

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>Rapamycin (25 mg/kg)</th>
<th>Rapamycin (100 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow cell per femur (x10^6)</td>
<td>20.7 ± 1.4</td>
<td>8.9 ± 1.5</td>
<td>18.8 ± 1.0</td>
</tr>
<tr>
<td>CFC per femur</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multilineage</td>
<td>749 ± 286</td>
<td>374 ± 195</td>
<td>356 ± 19</td>
</tr>
<tr>
<td>Erythroid</td>
<td>1,079 ± 203</td>
<td>2,879 ± 522</td>
<td>2,481 ± 646</td>
</tr>
<tr>
<td>GM</td>
<td>7,970 ± 362</td>
<td>9,369 ± 1,014</td>
<td>9,448 ± 2,083</td>
</tr>
<tr>
<td>Proliferative capacity per femur (cpm x 10^6)</td>
<td>5.7 ± 0.9</td>
<td>6.0 ± 0.9</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>Spleen wt (mg)</td>
<td>71.0 ± 2.7</td>
<td>58.4 ± 2.9</td>
<td>49.6 ± 3.1</td>
</tr>
<tr>
<td>Thymus wt (mg)</td>
<td>56.2 ± 4.6</td>
<td>30.9 ± 2.8</td>
<td>38.1 ± 5.3</td>
</tr>
</tbody>
</table>

C57Bl/6 mice were treated daily orally with rapamycin (25 and 100 mg/kg) or placebo on day 0 to 9, and killed on day 10. Results are pooled from two independent experiments with four mice per group each (mean ± SEM). In the colony assays, marrow cells were stimulated with a combination of IL-3, IL-11, and Epo as described in Materials and Methods.
unaffected by the rapamycin treatment, some lymphoid cells were decreased. The absolute number of bone marrow pre-B cells, characterized by their B220+ IgM+ phenotype, was consistently decreased by 50%, and the B220+ IgM+ B cells were decreased by 30% (W. Schuler, unpublished results).

Further long-term experiments in which rapamycin was given either at 25 mg/kg orally on alternate days for 3 weeks or at 50 mg/kg orally daily for 4 weeks gave similar results: there was no inhibition of bone marrow cellularity, CFC content, or proliferative capacity, whereas the spleen and the thymus were profoundly involuted and thymic cellularity was decreased (Table 2). Thus, normal mice treated for up to 28 days with doses of rapamycin that affect the lymphoid compartment do not manifest myelodepression.

Rapamycin prevents hematopoietic recovery after myelodepression in vivo. A single injection of 5-FU (150 mg/kg, IV) in mice causes a decrease in the number of CFC and pre-CFC by 95% and in the total bone marrow cell number by half after 2 days. Cellularity further decreases to less than 5% of normal numbers by day 5 after 5-FU injection. The hematopoietic stem cells are then recruited to cycle, and most CFC and pre-CFC numbers return to normal on day 8, with some overshoot on day 10 post-5-FU, which is accompanied by a recovery of bone marrow cellularity to 20% of normal. We used these experimental conditions to study the effect of rapamycin on recovering bone marrow hematopoiesis. Mice received one injection of 5-FU either on day 6 or day 0, and were killed on day 10 to control the state of myelodepression 4 days post-5-FU and the hematopoietic recovery 10 days post-5-FU (Fig 4). Another group of animals injected with 5-FU on day 0 received in addition rapamycin 50 mg/kg/d, orally daily for 10 days starting at day 0. Control mice were left entirely untreated or received rapamycin on days 0 to 9, but not 5-FU. As expected, the total bone marrow cellularity was reduced to 5% of normal values 4 days after 5-FU treatment and increased to 20% of the normal value by day 10 post-5-FU (Fig 4A). The number of CFC was also strongly decreased 4 days post-5-FU, and returned to almost normal values on day 10 post-5-FU (Fig 4B). Rapamycin strongly inhibited this

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

**Fig 4.** In vivo effect of rapamycin on the hematopoiesis of myelodepressed mice. Groups of four to six mice were left either untreated, or injected once with 5-FU on day 0 or day 6 of the experiment. The animals received no further treatment, or rapamycin (50 mg/kg/d orally) daily on day 0 to 10, as indicated. Mice were killed and the bone marrow cells harvested on days 10 and 17. The bone marrow cellularity (A, C) and the number of multilines (B), erythroid (B), and GM (x10; D) CFC per femur (B, D) were measured on day 10 (A, B) and on day 17 (C, D). The results are the mean ± SEM of two independent experiments (n = 8 to 11 mice per group on day 10 and n = 4 to 7 per group on day 17).
increase of bone marrow cellularity and CFC numbers between 4 to 10 days after 5-FU injection (Fig 4A and B). This effect was reversible because all parameters were essentially back to normal 7 days after cessation of rapamycin treatment (Fig 4C and D). Thus, rapamycin inhibits the hematopoietic recovery of myelocompromised animals under conditions where it does not affect normal myelopoiesis.

Dose-dependent effect of rapamycin on hematopoietic recovery. The dose response of rapamycin on hematopoietic recovery was investigated in the model described above. Rapamycin at 10 mg/kg inhibited the increase in bone marrow cell number between 4 and 10 days after 5-FU injection to 62% of the maximal effect obtained with 50 mg/kg rapamycin (Table 3). A slight inhibition was also seen with 2 mg/kg and 0.4 mg/kg rapamycin, but this was not significantly different from the controls. The effect of rapamycin on peripheral lymphoid organs was dose-dependent, as shown for thymus cellularity (Table 3). A similar dose response was found for the immunosuppressive activity of rapamycin determined in allogeneic vascularized heart transplantation using C57BI/6 mice as recipients, the same strain as in the 5-FU model, and Balb-c mice as donors (Table 3). Thus, the effect of rapamycin in preventing hematopoietic recovery of myelodepressed mice paralleled its immunosuppressive activity tested in the same strain of mice.

Increased cell proliferation after rapamycin withdrawal. After discontinuation of rapamycin treatment, bone marrow cell populations from mice treated with 5-FU plus rapamycin (50 mg/kg/d on day 0 to 9) manifested a higher proliferative capacity than those from control mice receiving 5-FU only (Table 4). When cultured for 3 to 4 days in the absence of exogenous growth factors, bone marrow cells harvested at day 10, after combined treatment with 5-FU and rapamycin, showed a ten-fold higher proliferation compared with cells from mice treated with 5-FU only (Table 4). This corresponded to a fourfold to 10-fold higher absolute proliferative capacity per femur in rapamycin-treated versus rapamycin-untreated mice. The proliferation of the same bone marrow cells could be further stimulated with cytokines present in WEHI and L929 cell-conditioned media, and the response was also 1.6-fold higher in the cells from rapamycin-treated mice. In addition, extramedullary hematopoiesis observed in spleen sections 17 days after 5-FU injection was dramatically increased 7 days after cessation of the rapamycin treatment. Splenic weight, total cellularity, and CFC content were also twice higher on rapamycin withdrawal in these animals compared with the 5-FU-treated controls (Fig 5).

DISCUSSION

We show here that rapamycin blocks the cytokine-driven in vitro proliferation of various hematopoietic cell lines, as well as primary cells. This was found for all cytokines tested, including most of the major hematopoietic growth factors such as IL-1, IL-3, IL-6, IL-11, G-CSF, GM-CSF, or KL. Likewise, rapamycin inhibited the IL-7-driven development of pre-B and B cells in long-term in vitro bone marrow cultures (W. Schuler, unpublished results). Rapamycin is not simply a cytotoxic drug. This is also evident from our experiments because the inhibitory effect of rapamycin on the proliferation of cell lines, and primary bone marrow cells can be antagonized by excess of FK-506, a structural analog of rapamycin that binds to the same intracellular proteins.

Assuming that cytokines are the essential driving force for maintaining steady-state hematopoiesis, and given the rapid turnover of bone-marrow cells, one would anticipate that in vivo rapamycin treatment for more than 10 days would result in a drastic reduction of bone marrow cellularity. This was not the case. Although the treatment of normal mice with rapamycin at doses that are immunosuppressive adversely affected thymus and spleen, it had no obvious effect on bone marrow cellularity, proliferative capacity, or number of myeloid progenitors, even after 4 weeks of treatment. This finding is in agreement with in vivo data in the rat, where bone marrow showed a normal histologic appearance after 14 days of treatment with rapamycin at 1.5

| Table 2. Effect of Long-Term Rapamycin Treatment on Hematopoiesis in Normal Mice |
|---------------------------------|-----------------|-----------------|
| Parameter                       | Placebo         | Rapamycin, 25 mg/kg/2 d (21 d) | Rapamycin, 50 mg/kg/d (28 d) |
| Bone marrow cells per femur (x10^6) | 13.9 ± 1.4     | 14.0 ± 3.1      | 22.9 ± 1.2       |
| CFC per femur                   |                 |                 |                 |
| Multilineage                    | 528*            | 1,038*          | 1,044 ± 102     |
| Erythroid                       | 1,479*          | 1,915*          | 3,264 ± 450     |
| GM                              | 11,624*         | 12,480*         | 11,301 ± 763    |
| Proliferative capacity per femur (cpm x10^6) | 12.3 ± 0.5     | 10.1 ± 2.0      | 7.0 ± 0.3       |
| Spleen weight (mg)              | ND              | 74.5 ± 0.5      | 57.8 ± 1.5      |
| Thymus weight (mg)              | 37.8 ± 2.0      | 29.8 ± 4.2      | 56.1 ± 3.2      |
| Cells per thymus (x10^6)        | ND              | 186.7 ± 18.6    | 83.3 ± 0.5      |

In experiment 1, Balb/c mice (n = 3 per group) were treated orally with placebo or rapamycin (25 mg/kg) every second day on day 0 to 20 and killed on day 21. In experiment 2, C57BI/6 mice (n = 4 per group) received placebo or rapamycin (50 mg/kg) orally daily on day 0 to 27 and were killed on day 28. In the colony assays, marrow cells were stimulated with a combination of IL-3, IL-11, and Epo as described in Materials and Methods. Mean ± SEM of results obtained with individual mice are given, except when indicated (*), where bone marrow cells from three mice were pooled.

Abbreviation: ND: not done.

From www.bloodjournal.org by guest on August 30, 2017. For personal use only.
Table 3. Dose-Dependency of the Effects of Rapamycin on In Vivo Hematopoietic Recovery and Allogeneic Heart Transplantation Survival

<table>
<thead>
<tr>
<th>Rapamycin Treatment (mg/kg/d)</th>
<th>Recovery 10 Days Post-5-FU*</th>
<th>Bone Marrow Cell/Femur (×10^6)</th>
<th>Thymus Cellularity (×10^6)</th>
<th>Vascularized Heart Allograft Survival (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.3 ± 0.6</td>
<td>32.2 ± 7.5</td>
<td>6.1 ± 0.2 (n = 10)</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>3.9 ± 0.7</td>
<td>28.6 ± 4.4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.5 ± 0.9</td>
<td>17.4 ± 5.6</td>
<td>8.7 ± 0.3 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.8 ± 0.6b</td>
<td>6.4 ± 2.1a</td>
<td>13.0 ± 2.1 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.0 ± 0.2</td>
<td>0.95 ± 0.1</td>
<td>21.0 ± 3.6 (n = 3)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*C57Bl/6 mice received placebo or rapamycin orally daily at the dose indicated on day 0 to 9. The animals were killed and the bone marrow cells enumerated on day 10. Results are pooled from two independent experiments with four mice per group each (mean ± SEM).

†Untreated controls had 22.3 ± 2.6 × 10^6 cells per femur and it decreased to 1.5 ± 0.2 × 10^6 cells per femur 4 days after 5-FU injection.

‡Thymus cellularity was 150.0 ± 21.0 and 6.5 ± 1.1 × 10^6 cells per thymus in untreated controls and 4 days post-5-FU, respectively.

§C57Bl/6 mice were transplanted with vascularized Balb/c heart and received rapamycin daily (orally) at the dose indicated. Graft function was assessed daily by palpation. Rejection was concluded when graft stopped beating and was confirmed by histology.

1 Level of significance, P < .01 using t test.

mg/kg/d, intraperitoneally. Possible problems of bioavailability of rapamycin to the bone marrow, although not formally excluded, are difficult to conceive in such a vascularized organ, which is usually among the first targets for drug toxicity. In addition, we observed that lymphoid populations within the bone marrow, namely, pre-B and B cells, were consistently decreased in normal mice treated with rapamycin.

In contrast to normal marrow, the same immunosuppressive doses of rapamycin strongly inhibited the recovery of bone marrow myeloid progenitor and mature cells, after myelodepletion by 5-FU. Thus, the rapamycin-sensitive cytokine pathways seem to be essential for hematopoietic recovery, but not for steady-state hematopoiesis. This might indicate that cytokine-driven pathways are indispensable in situations where recruitment of new precursors from the quiescent stem cell pool is needed, but that they are not generally essential for the maintenance of steady-state hematopoiesis. Alternatively, it is conceivable that in stress situations, such as recovery after myelodepression or in vitro culture, hematopoietic precursors employ an alternate signaling pathway that would involve the molecular target of rapamycin. That different regulatory mechanisms might be involved in stress situations is supported by previous studies using Macrophage inflammatory protein-1α (MIP-1α), a negative regulator of hematopoiesis. MIP-1α was shown to inhibit the proliferation of spleen colony-forming cells (CFU-S) from regenerating marrow in vitro and in vivo. Like rapamycin, MIP-1α prevents the recovery of CFC and pre-CFC after S-FU injection, although these progenitors are not sensitive to MIP-1α in normal mice.

The mode of action of rapamycin is not yet understood. Rapamycin binds to an intracellular binding protein, FKBP (FK-506 binding protein), and it is this complex that interferes with the progression through the cell cycle, arresting the cells in the late G1 phase. One very early event observed in the signaling cascade, following the interaction of growth factor receptors with their respective ligands, is protein tyrosine phosphorylation. Growth factor receptors are either endowed with intrinsic tyrosine kinase activity, like c-kit, or associated with cytoplasmic tyrosine kinases such as p56lck in the case of the IL-2 receptor. Our results show that the effect of rapamycin is independent of the type of cytokine receptor involved, suggesting that rapamycin acts downstream of these early protein tyrosine phosphorylation events. Rapamycin was shown to inhibit the pathway leading to the cytokine-induced phosphorylation, and thus activation, of the p70 S6 kinase. The relevance of the above finding, and the importance of S6 phosphorylation for S-phase entry, is still debated. For instance, in the continuously growing IL-2-dependent T-cell line Kit225, rapamycin inhibited p70 S6 kinase activation, although it did not block cell proliferation. Proliferation of Kit225 cells was blocked by rapamycin when the cells were in a resting state, after being deprived of IL-2 for 2 to 3 days, before IL-2 stimulation. The difference in rapamycin activity observed in vivo in steady-state versus recovering hematopoiesis could thus be another manifestation of the failure of rapamycin to block proliferation once resting cells have entered the cell cycle.

Table 4. Ex Vivo Proliferation in the Absence of Exogenous Growth Factors of Bone Marrow Cells From Mice Treated With Rapamycin (50 mg/kg/d) for 10 Days After 5-FU Injection

<table>
<thead>
<tr>
<th>In Vivo Treatment</th>
<th>cpm per 50,000 Cells</th>
<th>cpm per Femur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>5-FU</td>
</tr>
<tr>
<td>Exp 1</td>
<td>214</td>
<td>2,360</td>
</tr>
<tr>
<td>Exp 2</td>
<td>81</td>
<td>113</td>
</tr>
<tr>
<td>Exp 3</td>
<td>70</td>
<td>1,128</td>
</tr>
<tr>
<td>Exp 4</td>
<td>184</td>
<td>1,442</td>
</tr>
</tbody>
</table>

C57Bl/6 mice were either untreated or received one injection of 5-FU on day 0 with or without further daily treatment with rapamycin (50 mg/kg/d orally) on day 0 to 9. All animals were killed on day 10, and their bone marrow harvested. Bone marrow cell culture (5 × 10^6 cells/microwell) was performed in RPMI containing 10% FCS without further addition of growth factors. Proliferation was measured after 3 (experiments 1 through 3) or 4 (experiment 4) days in culture. Results are the mean of four individual mice per group tested in triplicate.
to hematopoietic cytokines contained in conditioned media (B), and the proliferation of a variety of continuously growing cell lines of different origins. Rapamycin was also recently shown to interfere with the expression and/or activation of several cyclins and cyclin-dependent kinases that are involved in the regulation of the G1 phase of the cell cycle.\textsuperscript{40-54} Cell proliferation during hematopoietic recovery or under cell culture conditions may depend on the rapamycin-sensitive expression of a certain cyclin. Under steady-state conditions, the expression of such a cyclin might be regulated differently, thus rendering the cells less sensitive to rapamycin.

After myelodepletion by 5-FU treatment, the recruitment and proliferation of new precursor cells from the stem cell pool leads not only to bone marrow recovery, but also to massive splenic myelopoiesis on day 17. This stimulation appears to be further increased on rapamycin withdrawal in mice that received 5-FU injection followed by a 10-day rapamycin treatment. The proliferative capacity of bone marrow cells harvested on day 10 after combined treatment with 5-FU and rapamycin, and tested ex vivo was 10-fold higher than after 5-FU treatment alone. This was not just a relative enrichment of the proliferating cells in the marrow, because the absolute proliferative capacity per femur was also increased. The number of hematopoietic progenitors did not seem to account for this increased proliferation because there were twofold to threefold less CFC per femur in the rapamycin-treated animals than in the 5-FU controls. On the other hand, the growth requirements of the bone marrow cell population from rapamycin-treated mice were changed, as the cells did not need addition of exogenous growth factor to proliferate in vitro. In addition, the splenic hematopoiesis that is normally seen 17 days post-5-FU was also increased 7 days after rapamycin withdrawal in mice that received rapamycin on day 0 to 9 post-5-FU. The absolute spleen weight, cellularity, and CFC content were twofold higher in these mice compared with 5-FU controls. At this point, we cannot formally distinguish whether the increased proliferative capacity of the myeloid cell populations on rapamycin withdrawal is due to a higher number of proliferating cells or to an increased proliferative capacity per cell. Further investigations will be required to address this point.

In conclusion, the results presented here demonstrate that, although rapamycin blocks the response to hematopoietic cytokines in vitro, a sustained in vivo treatment with immunosuppressive doses of rapamycin for up to 4 weeks is not myelotoxic in normal mice. Rapamycin has an inhibitory effect on hematopoietic recovery after myelodepletion by 5-FU, and this is seen at doses that are immunosuppressive in heart allogeneic transplantation. This suggests that the effect on myelopoietic recovery is not a side effect of the rapamycin treatment, but rather another manifestation of the in vivo activity of rapamycin on cytokine pathways. Finally, our results indicate that the use of rapamycin as an immunosuppressant may be detrimental in myelocompromised individuals. Studies are under way to analyze the effect of rapamycin on hematopoietic reconstitution of lethally irradiated mice with syngeneic bone marrow.

\textbf{ACKNOWLEDGMENT}

We are grateful to G. Poschmann, S. Bolliger, M. Tanner, D. Meyer, and E. Andersen for their skillful technical assistance; to Dr M. Schulz and A. Garvin (Preclinical Research, Sandoz Pharma) and to Prof B. Ryffel (University of Zurich, Switzerland) for critical reading and discussion of the manuscript, and to Dr M. Wiles (Institute for Immunology, Basel, Switzerland), Dr G. Keller (National Jewish Center for Immunology, Denver, CO), Dr S.C. Clark (Genet-
ics Institute, Cambridge, MA), and Dr E. Liehl (Sandoz Research Center, Vienna, Austria) for fruitful discussions.

REFERENCES

12. Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoft SJ, Crabtree G, Schreiber SL: Two distinct signal transmission pathways in T lymphocytes are inhibited by alternative complexes formed between an immunophilin and either FK506 or rapamycin. Proc Natl Acad Sci USA 87:9231, 1990

38. Calvo V, Crews CM, Vik TA, Bierer BE: Interleukin 2 stimulation of p70 S6 kinase activity is inhibited by the immunosuppresant rapamycin. Proc Natl Acad Sci USA 89:7571, 1992


The immunosuppressant rapamycin blocks in vitro responses to hematopoietic cytokines and inhibits recovering but not steady-state hematopoiesis in vivo

VF Quesniaux, S Wehrli, C Steiner, J Joergensen, HJ Schuurman, P Herrman, MH Schreier and W Schuler