Rapamycin Protects Mice from Staphylococcal Enterotoxin B-Induced Toxic Shock and Blocks Cytokine Release In Vitro and In Vivo

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Staphylococcal enterotoxins are potent activators for human T cells and cause lethal toxic shock. Rapamycin, an immunosuppressant, was tested for its ability to inhibit staphylococcal enterotoxin B (SEB)-induced activation of human peripheral blood mononuclear cells (PBMC) in vitro and toxin-mediated shock in mice. Stimulation of PBMC by SEB was effectively blocked by rapamycin as evidenced by the inhibition of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), IL-6, IL-2, gamma interferon (IFN-γ), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and T-cell proliferation. In vivo, rapamycin protected 100% of mice from lethal shock, even when administered 24 h after intranasal SEB challenge. The serum levels of MCP-1 and IL-6, after intranasal exposure to SEB, were significantly reduced in mice given rapamycin versus controls. Additionally, rapamycin diminished the weight loss and temperature fluctuations elicited by SEB.

Based on the potent immunosuppressive effects of rapamycin, we investigated the therapeutic impact of rapamycin on SEB-mediated toxic shock. The therapeutic efficacy of rapamycin in SEB-induced toxic shock was investigated by using a lethal murine model with intranasal delivery of SEB (22). This “double-hit” murine model relies on two low doses of SEB without the use of sensitizing agents such as lipopolysaccharide (LPS) or galactosamine to induce lethal shock (6, 27, 33, 37, 45). In this “SEB-only” toxic shock model, SEB was administered intranasally (i.n.) and another dose of SEB was strategically given intraperitoneally (i.p.) 2 h later to induce systemic cytokine release and pulmonary inflammation with lethality as an endpoint. We examined the effect of rapamycin on proinflammatory cytokines and chemokines induced by SEB in vitro using human peripheral blood mononuclear cells (PBMC) as a first step to test its immunological effects on SEB activation.
MATERIALS AND METHODS

Reagents. Purified SEB was obtained from Toxin Technology (Sarasota, FL). The endotoxin content of these preparations was <1 ng of endotoxin/mg protein, as determined by the Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD). Human cytokine enzyme-linked immunosorbent assay (ELISA) kits and assay reagents were purchased from R&D Systems (Minneapolis, MN). Mouse cytokine ELISA reagents were obtained from Pharmingen (San Diego, CA). Rapamycin and all other reagents were from Sigma (St. Louis, MO).

Cell cultures. Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from healthy human donors. The PBMC (10^6 cells/ml) were cultured at 37°C in 24-well plates containing RPMI 1640 medium and 10% heat-inactivated fetal bovine serum. Cells were stimulated with SEB (200 ng/ml) for 16 h, and the supernatants were harvested and analyzed for tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), IL-6, IL-2, gamma interferon (IFN-γ), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), and MIP-1β by ELISA as described previously (25, 26). Rapamycin, when present, was added simultaneously with SEB.

Human T-cell proliferation assays. PBMC (10^3 cells/well) were plated in triplicate with SEB (200 ng/ml), with or without various concentrations of rapamycin, for 48 h at 37°C in 96-well microtiter plates. Cells were pulsed with 1 μCi/well of [3H]thymidine (New England Nuclear, Boston, MA) during the last 5 h of culture, as previously described (25). Cells were harvested onto glass fiber filters, and incorporated [3H]thymidine was measured by liquid scintillation. In experiments investigating the effectiveness of rapamycin after a short pulse, PBMC (7 × 10^5 cells/ml) were cultured with SEB (200 ng/ml), with or without rapamycin, for 3 h at 37°C in 4-ml polypropylene round-bottomed tubes. Cells were centrifuged at 1,200 × g for 10 min, and supernatants were removed at the end of 3 h. Cells (10^5 cells/well) were recultured with fresh medium without rapamycin and SEB and replated in triplicates for 45 h at 37°C in 96-well microtiter plates. Cells were pulsed with [3H]thymidine and harvested as described above.

Murine model of SEB-induced toxic shock. Male C3H/HeJ mice (National Cancer Institute, Frederick, MD), weighing ~20 g each (7 to 10 weeks old), were housed in conventional microisolator cages. Sterile temperature/identification transponders (IPTT-300; Biomedic Data Systems, Maywood, NJ) were implanted subcutaneously into each animal 5 to 10 days before SEB exposure, and temperatures were monitored twice daily. Initial weight of animals was recorded 3 to 7 days before SEB exposure, and weight changes were recorded once daily after SEB challenge. SEB was administered i.n. (50 μg) and i.p. (200 μg SEB/mouse, as this was the optimal timing for serum cytokine collection after mice were given a dual dose of SEB (22). Sera were collected from anesthetized mice by cardiac puncture at 5 and 24 h after SEB exposure. Rapamycin (0.7 mg/kg) was administered at 2 and 5 h after intranasal SEB administration. Serum cytokine/chemokine levels from individual mice (n = 5 mice per time point), were analyzed for cytokines/chemokines using specific ELISA as described previously (22, 27, 39). Background levels of each cytokine/chemokine, all found to be negligible, were derived from a prebleed of the same mice performed 2 to 4 days before each experiment. For mice receiving delayed treatment with rapamycin at 24 h, whole lungs (n = 5) were excised from euthanized animals at 51 h post-toxin challenge, with and without treatment with rapamycin; weighed; and frozen at −70°C. Immediately before cytokine assays, lungs were thawed and homogenized for 30 min at 4°C in lysis buffer (Cell Signaling Technology, Danvers, MA) to a 7-mg/ml concentration with complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Homogenates were centrifuged at 500 × g for 10 min, and supernatants were harvested and then filtered through a 0.45-μm membrane (Millipore, Bedford, MA). Supernatants from lung homogenates were assayed for MCP-1, IL-1β, and IL-6 by ELISA as described above.

Cytokine detection. The levels of human cytokines (TNF-α, IL-1, IL-6, IL-2, and IFN-γ) and chemokines (MCP-1, MIP-1α, and MIP-1β) in culture supernatants from PBMC or murine mediators (TNF-α, IL-1β, IL-6, IL-2, IFN-γ, and MCP-1) in serum were measured via a sandwich ELISA by using cytokine-specific antibodies according to the manufacturer’s instructions (25–27, 39). Recombinant cytokines (20 to 1.000 pg/ml) represented the standards for calibration, and the detection limit of all assays was 20 pg/ml.

Determination of rapamycin in murine whole blood. Blood was collected from rapamycin-treated mice (n = 5) by retro-orbital bleeding into EDTA tubes 1 h after intranasal administration of rapamycin. Blood was centrifuged at 12,000 × g for 3 min to remove cells. Supernatants were removed and stored at −70°C until analysis. Analysis of rapamycin was performed as previously described. Sera were collected from anesthetized animals using an Agilent Technologies series 1100 capillary high-pressure liquid chro-matography (HPLC) instrument and an ion-trap mass spectrometer.

Statistical analysis. The cytokine data were expressed as the mean ± SD and analyzed for significant differences by the Student t-test with Stat software (Stata Corp.). Statistical comparisons of survival data were performed by Fisher’s exact test with Stata software (Stata Corp.). Differences were considered significant if P was <0.05.

RESULTS

Rapamycin inhibits SEB-induced cytokines from human PBMC. Because proinflammatory cytokines mediate the lethal effects of SEB, the effect of rapamycin on the production of these mediators was examined on human PBMC incubated with SEB. Figure 1 shows that rapamycin effectively blocked in a dose-dependent manner the production of TNF-α, IL-1β, IL-6, IL-2, and IFN-γ from PBMC incubated with SEB, achieving inhibition levels of 77%, 97%, 67%, 100%, and 100%, respectively, at 6.9 μg/ml of rapamycin compared to controls incubated with toxin alone (P < 0.05). The effect of rapamycin was more pronounced on T-cell cytokines, IL-2 and IFN-γ. At 6.9 ng/ml rapamycin, IL-2 and IFN-γ were suppressed by 34% and 36%, respectively, compared to cells stimulated with SEB alone (P < 0.05). Rapamycin also dose-dependently reduced the production of chemokines MCP-1, MIP-1α, and MIP-1β from SEB-stimulated PBMC. This indicates that rapamycin inhibited both cytokines and chemokines produced in vitro by both T cells and monocytes in response to SEB.

Rapamycin inhibits SEB-induced human T-cell proliferation. In addition to increasing cytokine levels, SEB also stimulates T-cell proliferation. Therefore, the effect of rapamycin...
on SEB-induced proliferation of T cells was examined next. The results showed that rapamycin significantly decreased SEB-induced proliferation of T cells in a dose-response manner, with maximal inhibition (94%) achieved at the same concentration (6.9 μg/ml) that was most effective at blocking cytokine and chemokine release (Fig. 2). At 0.69 μg/ml rapamycin, T-cell proliferation was reduced by 56%. The lack of proliferation or cytokine release from PBMC was not due to the cytotoxic effect of rapamycin, as determined by trypan blue exclusion test (data not shown).

We also investigated whether a short pulse of rapamycin was sufficient to block SEB-induced T-cell proliferation. When rapamycin was removed after 3 h of incubation, T-cell proliferation was decreased by 40% with 6.9 μg/ml of rapamycin. Thus, partial inhibition of proliferation was obtained when rapamycin was present for a short period of 3 h.

Rapamycin protects mice from SEB-mediated shock. Since rapamycin was effective in attenuating the biological effects of SEB in vitro, we examined its efficacy in vivo. Table 1 shows that rapamycin significantly increased the survival rate among

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<th>Toxin</th>
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*The percentage of mice alive 168 h after intranasal SEB exposure. Rapamycin (0.7 mg/kg) was given i.n. at the designated time points after SEB exposure. All rapamycin treatment groups (n = 10) also received dexamethasone (5 mg/kg) i.p. 24, 48, 72, and 96 h after SEB exposure. Dexamethasone (1.2 mg/kg) was given i.n. at the designated time points after SEB exposure. All dexamethasone-treated groups (n = 10) also received dexamethasone (5 mg/kg) i.p. 24, 48, 72, and 96 h after SEB exposure. There were no lethal effects among mice given PBS or BSA. ND, not determined.
mice given two doses of SEB. We progressively increased the time between the exposure of mice to SEB and rapamycin treatment and found that mice were protected 100%, even when rapamycin was given 5 h after the SEB exposure (Table 1). Clinical signs of intoxication such as ruffled fur and lethargy observed with SEB-treated mice starting at 72 h were completely absent from the SEB-plus-rapamycin group. Additional data were collected regarding temperature fluctuations in mice treated with SEB and those treated with SEB plus rapamycin given at various times after SEB exposure. Mice given SEB experienced hypothermia starting at 48 h (Fig. 3). This hypothermic response, indicating systemic shock that mimicked those found in other murine models, was completely absent in rapamycin-treated, SEB-exposed mice. Rapamycin protected mice from systemic shock even when it was administered 5 h after SEB exposure, as seen from the normal temperature of rapamycin-treated animals after intranasal delivery of SEB. SEB-exposed mice experienced weight loss of 5% at 77 h whereas rapamycin-treated, SEB-exposed mice had weight gains of 8 to 90% and 80%, respectively, in SEB-plus-rapamycin-treated mice. Therefore, the in vivo concentration for effective treatment as previously described (18). The blood level of rapamycin 1 h after an i.n. dose of 0.7 mg/kg was 492 ± 204 ng/ml. At 97 h after multiple dosing (i.n. doses of 0.7 mg/kg at 2 and 5 h followed by i.p. doses of 1.6 mg/kg at 30, 48, 72, and 96 h), rapamycin reached 622 ± 282 ng/ml. The latter concentration likely represented the highest concentration of rapamycin achieved in this dosing regimen, as the concentration of rapamycin reportedly peaked 1 h after dosing (49). In contrast, the blood concentration of rapamycin at 97 h dropped to 38 ± 15 ng/ml after the use of lower doses of rapamycin (0.03 mg/kg i.n. at 2 and 5 h and 0.08 mg/kg i.p. at 24, 48, 72, and 96 h) which were totally unprotective against SEB-mediated shock.

We also compared the efficacy of rapamycin against that of a potent immunosuppressant, the corticosteroid dexamethasone. Dexamethasone was 100% effective in preventing lethality when mice (n = 10) were treated with i.n. dexamethasone at 2 and 5 h, followed by daily i.p. doses at 24, 48, 72, and 96 h (Table 1). However, delaying treatment with dexamethasone until 5 h after SEB exposure resulted in 90% lethality. Thus, rapamycin is a more effective therapeutic post-SEB exposure than dexamethasone in vivo (Fig. 5).

**Rapamycin attenuates serum levels of IL-6 and MCP-1 in vivo.** Based upon the strong inhibitory effects of rapamycin on SEB-mediated T-cell proliferation and cytokine production in vitro, the potential therapeutic role of rapamycin in vivo was further investigated in mice. Previous studies showed that elevated serum levels of MCP-1, IL-6, and IL-2 are a prominent feature of SEB-mediated toxic shock with intranasal delivery of SEB. Therefore, the in vivo effect of rapamycin on serum cytokine concentrations was examined in mice after SEB administration. Peak levels of MCP-1 and IL-6 were reduced by 90% and 80%, respectively, in SEB-plus-rapamycin-treated
mice versus SEB controls without rapamycin (Fig. 6). Serum concentrations of IL-2 remained unexplainably high in contrast to the in vitro inhibitory effect of rapamycin on IL-2 in SEB-stimulated human PBMC. We further examined serum IL-2 concentrations at later time points. There was no IL-2 present at 24, 51, and 75 h in both SEB- and SEB-plus-rapamycin-treated mice (data not shown). Thus, the elevated serum IL-2 at 5 h was transient with rapamycin treatment and did not affect lethality or the overall well-being of animals based on temperature and weight changes.

Since rapamycin was also effective when administered i.n. at 24 h after SEB exposure, lungs were excised at 51 h post-SEB challenge for evaluation of the effect of rapamycin on lung cytokines. Lung MCP-1, IL-2, and IL-6 were attenuated by 56%, 80%, and 65%, respectively, in mice treated with rapamycin compared to the SEB-exposed group not treated with rapamycin.

**DISCUSSION**

The present study demonstrates for the first time that rapamycin effectively inhibited SEB-mediated production of TNF-α, IL-1β, IL-6, IL-2, IFN-γ, MCP-1, MIP-1α, and MIP-1β by human PBMC in vitro. Besides decreasing the levels of proinflammatory cytokines in vitro, rapamycin also completely blocked SEB-induced proliferation of T cells. T-cell cytokines, IL-2 and IFN-γ, were more sensitive to low concentrations (ng/ml) of rapamycin. As excessive release of cytokines mediates the pathogenic effects of SEB in vivo, the use of rapamycin, an immunosuppressant, was a seemingly logical choice for our studies in vivo. Our study is the first to demonstrate that rapamycin was 100% effective in protecting mice from SEB-mediated shock. Previous studies of drug treatment for SEB-mediated shock models indicate a very narrow therapeutic window of treatment with different types of inhibitors to reduce the biological effects of SEB (1, 6, 27, 51). As demonstrated in this study, the potent immunosuppressant dexamethasone was not protective when administered 5 h after SEB exposure but was protective when given at 2 h post-exposure to SEB. In a lipopolysaccharide (LPS)-synergized murine model of SEB-induced shock, “protective” peptides derived from conservative regions of SEB, even when given 1 h before SEB exposure, resulted in 83% protection (51). Our in vivo studies here indicate a wider therapeutic window for rapamycin and show that rapamycin, even when given 24 h after SEB exposure, still afforded 100% protection against mortality and temperature and weight fluctuations. Serum levels of MCP-1 and IL-6 were also markedly reduced in mice treated with rapamycin compared to control SEB-exposed mice. These results demonstrated the potency of rapamycin even when given post-SEB exposure. Clearly, the serum cytokine, temperature, and weight data revealed the protective effects of rapamycin after SEB exposure. Although we could not explain the high levels of IL-2 in vivo with rapamycin treatment, we found that this effect was transient and did not affect survival. Rapamycin given as late as 24 h also reduced cytokines and chemokines in lung tissues. Other effects of rapamycin on cells of the
immune system and mTOR pathway, not explored fully in this study, likely contributed to its potency.

The rapamycin doses used in vivo in this study were in the same range as those used previously to reduce murine arthritic arthritis and carcinogen-induced lung tumors (7, 18). Peak blood concentrations of rapamycin also agreed with those used in murine models (18). In healthy subjects, blood concentra-
tions of rapamycin reached 78 ± 18 ng/ml 1 h after adminis-
tration of 15 mg of rapamycin (49). Rapamycin is used for extended periods of years to prevent graft rejection in renal transplantation. In these patients, long-term accumulative toxic effects must be taken into consideration. The doses of rapamycin in the present study were similar to those used in animal models of disease (7, 18), and the short therapeutic course of treatment described in this study suggests that rapa-
mycin can be considered a good candidate for transition to clinical trials to treat SEB-induced shock. Obviously, different doses have to be tried and adjusted to limit toxicity in humans. Given the small dose discrepancy of rapamycin in previous mouse models of disease and similar disease in humans, lower doses of drug would likely be effective in treating SEB-induced shock in humans.

The pathophysiology and treatment of shock with Staphylo-
coccus aureus-secreting superantigens such as SEB and toxic shock syndrome toxin 1 are much more complex. Further studies are needed for animal model development to establish in vivo models that can separate effects of bacterial infection and toxic effects of many bacterial virulence factors, including super-
antigens. With the continuing rise of vancomycin- and methicillin-resistant Staphylococcus aureus strains, there is an urgent need for new antibacterials against drug-resistant microorganisms. A logical extension of this study is to apply rapamycin in an S. aureus infection model with bacteria producing SEB and other superantigens.

In conclusion, our in vivo studies showed that the beneficial effects of rapamycin in SEB-induced shock included increased survival, minimal temperature and weight change, and a dra-
matic reduction in the serum levels of MCP-1 and IL-6. These findings are significant because rapamycin can be given as late as 24 h after intranasal SEB exposure. Previous studies indi-
cate a good correlation between superantigen toxicity in mice and elevated levels of proinflammatory cytokines (6, 22, 33, 39, 45). The promising findings of this study make rapamycin a potential initiator of graft rejection in renal transplantation. In these patients, long-term accumulative toxic effects must be taken into consideration. The doses of rapamycin in the present study were similar to those used in animal models of disease (7, 18), and the short therapeutic course of treatment described in this study suggests that rapa-
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