Cyclosporin A and Cyclosporin SDZ PSC 833 Enhance Anti-CD5 Ricin A-Chain Immunotoxins in Human Leukemic T Cells

By Jean-Pierre Jaffrezou, Branimir L. Sikic, and Guy Laurent

Recent studies have shown that cyclosporin A (CsA) may affect ricin A-chain immunotoxin (RTA-IT) therapy. In this study, we evaluated the ability of CsA and its nonimmunosuppressive analog, SDZ PSC 833, to enhance anti-CD5 T101 RTA-ITs in vitro. Both 4 μmol/L CsA and 4 μmol/L SDZ PSC 833 significantly and specifically enhanced the cytotoxic activity of T101 RTA-IT on the human lymphoblastic T-cell line, CEM III (101-fold and 105-fold, respectively). Furthermore, these CsA also enhanced the cytotoxicity of the more potent T101 F(ab)2 RTA-IT (ninefold and eightfold, respectively). The effect of human plasma, originating from four patients enrolled in a phase I high-dose CsA regimen, was examined on T101 RTA-IT cytotoxicity on CEM III cells. In each case, with plasma CsA levels between 3,090 and 4,860 ng/mL (2.5 to 4 μmol/L), a significant increase in T101 RTA-IT–mediated cytotoxicity was observed ranging from 31% to 60%. Neither CsA nor SDZ PSC 833 affected the rate of RTA-IT binding, internalization, intracellular trafficking, or degradation. Analysis of internalized T101 RTA-IT molecules showed that these were essentially intact, which suggests that these enhancers may act only on a small population of RTA-ITs that escapes present investigational techniques. In conclusion, because the concentrations used are clinically achievable, CsA appear to be promising agents for in vivo enhancement of RTA-ITs.

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ENHANCEMENT OF ANTI-CD5 IMMUNOTOXINS

Hanover, NJ). These were dissolved in absolute ethanol at 1 mmol/L and stored at −20°C. All other reagents were purchased from Sigma (St Louis, MO).

Human plasma. Plasma samples were obtained from four patients enrolled in a phase I trial of high-dose CsA for modulation of multidrug resistance.24 Samples were collected before and 2 hours after CsA infusion. CsA levels were measured using a nonspecific fluorescence-polarization immunosassay that cross-reacts with both CsA and metabolites (TDX; Abbott Laboratories, North Chicago, IL) and found to be between 3,090 and 4,860 ng/mL.

Protein-synthesis inhibition assay. RTA-IT efficacy was evaluated by the inhibition of [3H]-Leucine incorporation as described previously.2 Briefly, 1 x 10^6 cells/mL in complete medium were treated during 18 hours at 37°C with various concentrations of toxin or RTA-IT and, when specified, in the presence of Cs. [3H]-Leucine (specific activity, 150 Ci/mmol; 1 µCi/100 µL well; Amersham, Arlington Heights, IL) was added 6 hours before the end of treatment. Washed cells were harvested on filter paper (Cambridge Technology, Inc, Watertown, MA) and radioactivity was counted. Results of triplicate experiments are expressed as the percentage of control (cells not treated by toxin or RTA-IT). In experiments using human plasma, 2 x 10^6 CEM III cells were pelleted and resuspended in 100% human plasma. After addition of 1 x 10^-4 mol/L T101 RTA-IT, the final plasma concentration was 87%.

121I labeling of T101 RTA-IT. T101 RTA-IT (50 µg) was labeled with 1 mCi of 121I (Amersham) in the presence of chloramine T.25 After 1 minute of incubation, the reaction was stopped by the addition of sodium metabisulfite followed by potassium iodide. Free 121I was removed by chromatography on a phosphate-buffered PD-10 Sephadex G-25 M column (Pharmacia, Piscataway, NJ). The collected fractions that contained labeled protein were pooled and the radioactivity counted. The specific radioactivity of labeled T101 RTA-IT was approximately 7.3 µCi/mg protein. Iodination did not modify the activity of T101 RTA-IT (data not shown).

Rate of T101 RTA-IT internalization in CEM III cells. The kinetics of T101 RTA-IT internalization was measured according to the previously described method.26,28 Briefly, CEM III cells were incubated for 1 hour at 4°C with 121I-labeled T101 RTA-IT (at 10-fold the membrane-saturable concentration: 1 x 10^-8 mol/L) in complete medium. A negative control was performed by incubating the cells at 4°C with cold T101 RTA-IT (1 x 10^-4 mol/L) 2 hours before addition of 121I-T101 RTA-IT. After three ice-cold washes with saline solution (removing excess 121I-RTA-IT), cells (1 x 10^6/mL) were incubated at 37°C, with or without 4 µmol/L CsA, for various incubation times. At the end of these times, cells were pelleted by centrifugation and washed twice. The radioactivity of the collected supernatants, which represented non–cell-associated 121I-ligand, represented less than 10% of the total recovered radioactivity. Nonendocytosed cell-surface bound 121I-ligand was removed by treatment for 30 minutes at 4°C with 0.4% (wt/vol) Streptomyces griseus protease (Sigma). Protease treatment removed greater than 90% cell-surface bound radioactivity. Cells were again centrifuged and the radioactivity in the cell pellet and supernatant determined. From these determinations, the amount of T101 RTA-IT endocytosed (Et) was calculated: Et = 100 x Pt/(Pt + St). Where Et = endocytosis at time t, Pt = counts/min in cell pellet at time t, and St = counts/min in supernatant (of protease-treated cells) at time t. Results are expressed as the percentage of total recovered radioactivity, which remained constant throughout the experiment. Radioactivity was determined with a gamma counter.

Analysis of T101 RTA-IT degradation in CEM III cells. After protease treatment, washed cells were lysed with 50 mmol/L Tris-HCl, 0.5% sodium dodecyl sulfate (SDS), pH 8. Intracellular non-degraded and degraded 125I-ligand were assessed by measuring the 10% trichloroacetic acid (TCA)-precipitable and soluble radioactivities, respectively.

Percol gradient fractionation of organelles. The intracellular trafficking of 125I-T101 RTA-IT in CEM III cells was evaluated according to the previously described method.27 With slight modifications. Cells (1 x 10^6) were incubated with 1 x 10^-4 mol/L 125I-T101 RTA-IT for 4 hours at 37°C with or without 4 µmol/L CsA or 4 µmol/L SDZ PSC 833. Cells were then washed twice in ice-cold phosphate-buffered saline and resuspended in 1 mL of cold buffer (250 mmol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L triethanolamine), and homogenized with 25 strokes of a Dounce homogenizer (Cole-Parmer Instrument Co, Niles, IL). After centrifugation at 250g for 10 minutes at 4°C to sediment nuclei and unbroken cells, the supernatant was recovered and carefully layered onto the surface of a tube containing 9 mL of 20% Percoll in cold buffer resting on a cushion of 0.5 mL 2.5 mol/L sucrose. Percoll gradients were generated by centrifugation at 20,000g for 90 minutes at 4°C. Fractions of 0.5 mL were collected from the top and the radioactivity counted.

Molecular-weight evaluation of internalized molecules. After 4 hours of incubation with 125I-T101 RTA-IT at 37°C, cell-surface radioactivity was eluted from CEM III cells by treatment with S. griseus protease as described above. Analysis of the molecular weight of internalized 125I-T101 RTA-IT was performed according to the previously described method.2 Cells (5 x 10^6) were solubilized for 30 minutes at 4°C in 3.5 mL of 0.5% Nonident-P40, 1% Triton X-100, and 0.5% sodium deoxycholate containing protease inhibitors phenylmethylsulfonyl fluoride (100 µg/mL), pepstatin A (1 µg/mL), leupeptin (2 µg/mL), and aprotonin (2 µg/mL). The lysates were obtained after centrifugation at 1,000g and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5% to 16% gradient gel.24 Gels were autoradiographed at −70°C on Kodak X-OMAT AR film with Du Pont Cronex Lightning-Plus intensifying screens (Eastman Kodak, Rochester, NY) and analyzed by densitometry.

RESULTS

Enhancement of RTA-IT activity by CsA. The ability of CsA and SDZ PSC 833 to enhance anti-CD5 RTA-ITs was evaluated by treating the human lymphoblastic T-cell line CEM III with various concentrations of the drugs and RTA-IT. Figure 1 illustrates the relatively weak cytotoxicity of T101 RTA-IT compared with native ricin (>50,000-fold). Nevertheless, specificity and cytotoxicity are superior to ricin A-chain alone (about 10-fold). Coincubation of T101 RTA-IT with either 4 µmol/L CsA or 4 µmol/L SDZ PSC 833 resulted in a significant increase in cytotoxicity (Table 1). Enhancement of T101 RTA-IT cytotoxicity was achieved within a narrow range of CsA concentrations. At 1 µmol/L neither CsA nor SDZ PSC 833 had a significant effect on T101 RTA-IT cytotoxicity. A significant effect of 20- to 30-fold was observed at 2 µmol/L for both Cs, which further increased to greater than 100-fold at 4 µmol/L (a nontoxic concentration). However, Cs toxicity reached about 20% at 6 µmol/L, which prevented their use at higher concentrations (data not shown).

T101 Fab(αb)2 RTA-IT cytotoxicity on CEM III was about 10-fold more potent than its whole-fragment counterpart. This RTA-IT was also enhanced by CsA and SDZ PSC 833. At 2 µmol/L both CsA and SDZ PSC 833 had a limited
effect of about twofold but this increased significantly to about 10-fold at 4 μmol/L. The enhancement effect of both CsA and SDZ PSC 833 was specific, because the toxicity of ricin A-chain alone and the control anti-CD19 RTA-IT was unaffected. Furthermore, neither T101 RTA-IT or T101 F(ab')2 RTA-IT showed any change in nonspecific toxicity on the irrelevant human Burkitt's lymphoma cell line, RAJ1 (data not shown).

**Table 1. Comparative RTA-IT Enhancement on CEM III Cell Line**

<table>
<thead>
<tr>
<th>RTA-IT</th>
<th>Drug</th>
<th>μmol/L</th>
<th>IC50 (μmol/L)</th>
<th>Enhancement</th>
</tr>
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<tr>
<td>T101</td>
<td>No drug</td>
<td>25.000 ± 5.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CsA</td>
<td>1.0</td>
<td>18.000 ± 2.000</td>
<td>1.4</td>
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<td></td>
<td></td>
<td>2.0</td>
<td>1.233 ± 2.516</td>
<td>20.3</td>
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<td></td>
<td></td>
<td>4.0</td>
<td>247 ± 50</td>
<td>101.2</td>
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<tr>
<td></td>
<td>PSC 833</td>
<td>1.0</td>
<td>19.333 ± 1.154</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>866 ± 1.527</td>
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<td>4.0</td>
<td>237 ± 55</td>
<td>105.5</td>
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<td>T101 F(ab')2</td>
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<td>2,366 ± 321</td>
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<tr>
<td></td>
<td>CsA</td>
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<td>4.0</td>
<td>253 ± 25</td>
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<td></td>
<td>PSC 833</td>
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<td>1,953 ± 117</td>
<td>1.2</td>
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<td>290 ± 36</td>
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<tr>
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<td></td>
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<td></td>
<td>CsA</td>
<td>4.0</td>
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<td>&gt;50,000</td>
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</tr>
<tr>
<td>Ricin</td>
<td>[A-chain]</td>
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<td>273,333 ± 25,166</td>
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<td>240,000 ± 40,000</td>
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<td>246,866 ± 45,092</td>
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CEM III cells were incubated with different concentrations of RTA-IT and drugs for 18 hours. Then protein synthesis was measured and IC50 values were determined. Values are mean of three independent experiments ± SD.

The cytotoxicity of T101 RTA-IT on CEM III cells was evaluated in a prospective experiment using human plasma (Fig 3). T101 RTA-IT, 1 × 10^{-8} mol/L, inhibited protein synthesis in the presence of four pre-CsA control-plasma samples by 43%, 52%, 62%, and 50%, respectively. In the presence of patient plasma obtained 2 hours post-CsA infusion24 T101 RTA-IT cytotoxicity increased to 61%, 77%, 85%, and 77%, respectively.

**Effect of CsA on internalization and degradation of ^125I-T101 RTA-IT.** CEM III cells were incubated with a membrane-saturating concentration of ^125I-T101 RTA-IT (1 × 10^{-8} mol/L) for 1 hour at 4°C. After washing in ice-cold saline solution (removing excess ^125I-RTA-IT), CEM III cells were incubated at 37°C; with or without 4 μmol/L CsA. At specified times, cells were pelleted, membrane-bound labeled ligand was removed by a protease treatment, and the radioactivity of the pellet was determined. Figure 4 shows the slow internalization rate of T101 RTA-IT in CEM III cells. Furthermore, treatment by 4 μmol/L CsA did not significantly modify the kinetics of internalization.

After protease treatment, CEM III cells were lysed and intracellular nondegraded and degraded ^125I-ligand was...
ENHANCEMENT OF ANTI-CD5 IMMUNOTOXINS

evaluated by measuring the 10% TCA-precipitable and soluble radioactivities, respectively. Figure 5 shows that the degradation of internalized T101 RTA-IT increased steadily until it reached approximately 30%. This implies that greater than 70% of the internalized T101 RTA-IT remained intact. Treatment with 4 μmol/L CsA had no significant impact on internalized ligand. In a control experiment, preincubation of CEM III cells for 1 hour at 37°C with 4 μmol/L CsA before 125I-T101 RTA-IT treatment did not modify the plasma-membrane binding characteristics of the ligand (data not shown).

Subcellular distribution of T101 RTA-IT. The intracellular trafficking of 125I-T101 RTA-IT in CEM III cells was evaluated by density-gradient centrifugation of lysed cells treated with 1 × 10^{-8} mol/L T101 RTA-IT for 4 hours at 37°C with or without 4 μmol/L CsA. The distribution of cell organelles within the gradient reflects the distribution of T101 RTA-IT within the cell, ie, Golgi and plasma mem-
were incubated for 4 hours at 37°C with $1 \times 10^{-8}$ mol/L $^{125}$I-T101 RTA-IT with or without 4 $\mu$mol/L CsA or 4 $\mu$mol/L SDZ PSC 833. The cells were then treated with protease to remove membrane-bound labeled ligand, solubilized in detergent, and analyzed by SDS-PAGE. Figure 7A and B show that no detectable changes in toxin/MoAb ratio were observed between untreated $^{125}$I-T101 RTA-IT and 4 $\mu$mol/L CsA or 4 $\mu$mol/L SDZ PSC 833. No free ricin A-chain was detected (data not shown).

**DISCUSSION**

Recently, two studies have reported an interaction of CsA in combination with RTA-IT therapy. Przepiorka et al\(^\text{11}\) noted that among allogeneic BM-transplantation patients who received an anti-CD5 RTA-IT regimen as graft-versus-host disease prophylaxis, the incidence of RTA-IT toxicity (capillary leak syndrome [CLS]) substantially increased in patients who also received conventional CsA immunoprophylaxis. More recently, Yefenof et al\(^\text{10}\) described that the combination of CsA and an RTA-IT directed against the radiation leukemia virus envelop glycoprotein (gp70) prevented the development of malignancy in mice inoculated with radiation leukemia virus-induced preleukemic cells. This report proposed that CsA increased the effectiveness of the RTA-IT regimen by blocking interleukin-4 (IL-4) secretion, thereby limiting preleukemic cell autostimulation.

In light of these findings, we investigated the potential of CsA to directly increase the cytotoxicity of RTA-ITs. The ability of CsA and its nonimmunosuppressive analog, SDZ PSC 833, to enhance the in vitro activity of anti-CD5 RTA-ITs on the human lymphoblastic T-cell line, CEM III was evaluated. The cytotoxicity of anti-CD5 T101 RTA-IT was evaluated by over 100-fold by 4 $\mu$mol/L CsA and 4 $\mu$mol/L SDZ PSC 833 in vitro. The F(ab)'2 fragment counterpart of the whole T101 Ig RTA-IT, although more potent\(^\text{4,5}\) was less...
enhanced, about 10-fold. In previous studies, we observed that other RTA-IT enhancers such as NH₄Cl, monensin, and calcium antagonists, were less active on RTA-IT fragments than on their whole-Ig counterparts.³⁴ We also observed that 4 μmol/L CsA increased the cytotoxicity of TI01 RTA-IT by 150-fold on the murine leukemia cell line T2, and both 4 μmol/L CsA and 4 μmol/L SDZ PSC 833 increased the cytotoxicity of an anti-CD19 RTA-IT by 10-fold on the human Burkitt's lymphoma cell line RAJI (data not shown).

The RTA-IT enhancement effect of CsA was specific because the toxicities of neither ricin A-chain alone nor the irrelevant anti-CD19 RTA-IT (and an anti–HLA-DR RTA-IT, data not shown) were affected in CEM III cells. Furthermore, the nonspecific toxicity of both anti-CD5 RTA-ITs was also unaffected on the irrelevant human Burkitt's lymphoma cell line RAJI.

We extended our investigation by evaluating the cytotoxicity of TI01 RTA-IT in CEM III cells incubated in human plasma. We have previously shown that the activity of potent RTA-IT enhancers, such as monensin and perhexiline, is inhibited by serum components.³⁹ At the membrane saturating concentration of 1 × 10⁻⁸ mol/L, mean TI01 RTA-IT protein-synthesis inhibition in four plasma samples was about 50%, which was not significantly different from that observed in regular culture media. However, the cytotoxicity of the RTA-IT was increased to an average of 75% when CEM III were incubated in patient plasma after high-dose CsA infusion.³⁵ The CsA levels in the plasma were between 3,090 and 4,860 ng/mL (2.5 to 4 μmol/L) as assessed by the immunoassay, TDX.

The fact that effective CsA levels can be achieved in vivo is a crucial observation. We have recently shown that high-dose CsA can be administered with acceptable toxicity.²⁴ Indeed, short-term infusion led to steady-state CsA levels up to 4 μmol/L, without long-term immunosuppressive consequences. Even though our in vitro experiments using patient's plasma were performed for 18 hours, the kinetics of cytotoxicity experiments, using the CEM III cell line, strongly indicated that shorter incubation times would also lead to significant enhancement of RTA-IT cytotoxicity. Moreover, prolonged exposure to both RTA-IT and CsA would be unnecessary because about 50% of the bound RTA-IT are already internalized in CEM III cells in little over 2 hours.

The mechanism by which drugs enhance RTA-IT cytotoxicity remains unclear. Certain agents such as the calcium antagonists perhexiline and SR33557 appear to act by blocking the intracellular degradation of RTA-ITs and profoundly modifying its routing.⁹,¹⁰ However, lysosomotropic amines, such as NH₄Cl, and carboxylic ionophores, such as monensin, do not alter either degradation or intracellular trafficking of internalized RTA-ITs.⁷,⁸ These agents do increase the kinetics of cytotoxicity.⁷,⁹,¹⁰,¹²

TI01 RTA-IT has a distinctive slow rate of cell intoxication,¹² and enhancers such as monensin and NH₄Cl are able to increase the rate of cell kill dramatically.⁷,¹² In our investigation, we found that both 4 μmol/L CsA and 4 μmol/L SDZ PSC 833 similarly increased the kinetics of protein synthesis inhibition of TI01 and TI01 F(ab')₂ RTA-ITs. Unlike the calcium antagonist, but much like monensin and NH₄Cl, CsA had no effect on membrane binding, internalization, intracellular degradation, or intracellular trafficking of TI01 RTA-IT. Hence, the mechanism of CsA on CEM III cells leading to RTA-IT enhancement remains unclear. Unlike the calcium antagonists, CsA did not have any inhibitory effect on lysosomal acid sphingomyelinase and electron-microscopy studies did not show the presence of osmiophilic-laminated structures (data not shown) that are indicative of the perturbation of the lipid metabolism observed in perhexiline and SR33557-treated cells.⁹,¹⁰ However, CsA has been noted as possibly binding to lipid domains and thereby provoking changes in both membrane polarity and lipid order.³⁰,³¹

Among the limitations of RTA-IT therapy, CLS appears to be the most important side effect.³²,³³ The recent report
that CLS substantially increased in patients who also received conventional CsA immunoprophylaxis may limit their combined use. However, CLS can be treated by glucocorticoids, and because CsA significantly enhances the rate of RTA-IT cytotoxicity, shorter RTA-IT treatment times may prove effective. CLS is a major side effect of IL-2 therapy, and may possibly be linked to the immunosuppressive activity of CsA. This would make the nonimmunosuppressive CsA analog, SDZ PSC 833, a more appropriate RTA-IT enhancer.

In conclusion, agents such as Cs could significantly improve the clinical potential of RTA-ITs by enhancing their cytotoxicity. Although animal studies are required to confirm our observations, CsA and its nonimmunosuppressive analog, SDZ PSC 833, appear to be promising agents for in vivo RTA-IT enhancement.

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JAFFREZOU, SIROC, AND LAURENT
Cyclosporin A and cyclosporin SDZ PSC 833 enhance anti-CD5 ricin A-chain immunotoxins in human leukemic T cells

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