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

By Jean-Pierre Jaffrezou, Branimir 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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IMMUNOTOXINS (ITs), which are composed of a monoclonal antibody (MoAb) coupled to a bactericidal toxin, are potentially highly cytotoxic and specific agents for the targeting of cancer cells. Ricin A-chain immunotoxins (RTA-ITs), for example, are among the most potent ITs being evaluated for their potential use as anticancer agents. T101 RTA-IT and T101 F(ab)2 RTA-IT directed against the lymphoid differentiation antigen, CD5, have been found in vitro to be both specific and cytotoxic to lymphoid leukemia cells.6,8 However, compared with the native ricin toxin, these RTA-ITs, especially T101 RTA-IT, present limited antitumor efficacy probably because of the low rate of cell intoxication and/or unfavorable intracellular trafficking.7,10

One of the most active protein inhibitors at the acellular level, ricin A-chain is comparable with other toxins of bacterial (diphtheria toxin and Pseudomonas exotoxin) and higher-plant (pokeweed antiviral protein and gelonin) origin.2 However, the absence of the ricin B-chain, which plays a role in facilitating the translocation of the A-chain from the endosome to the cytosol, greatly decreases the activity of RTA-ITs.11 Therefore, although these conjugates show stringent specific cytotoxicity, their clinical potential depends on ways of increasing their cytotoxic activity.

Potentiating of RTA-ITs, including T101 RTA-IT, cytotoxicity has been achieved in vitro with the use of agents such as carboxylic ionophores,1,2,13 lysosomotropic amines,12,14 and calcium antagonists.9,10,15-17 Some of these enhancers can be used ex vivo such as in bone marrow (BM) purging in humans,2 but most of them are ill-suited for in vivo use where the major obstacles are either their toxicity or short half-life after intravenous administration.18,19

Recent studies have suggested that cyclosporin A (CsA) increases the efficacy/toxicity of certain RTA-ITs.20,21 Therefore, we evaluated the potential RTA-IT enhancement effect of CsA and its nonimmunosuppressive analog, SDZ PSC 883. Both CsA and SDZ PSC 833 were found to be both potent and specific enhancers of T101 and T101 F(ab)2 RTA-IT in vitro. We also investigated the impact of these RTA-IT enhancers on the rate of T101 RTA-IT intoxication, binding, internalization, intracellular degradation, and subcellular localization.

MATERIALS AND METHODS

Cells. The human lymphoblastic T-cell line CEM III is permanently maintained in our laboratory by serial passage. CEM III was subcloned from CEM using a FACS IV cell sorter (Becton Dickinson, Mountain View, CA). These cells express a mean CD5 density of 4 pmol/L.101-F(ab)2 reacts with the cross-linking IgG2a antibody T101.22,23 Anti-CD19 MoAb, and ricin A-chain were purified and then conjugated by the cross-linking agent N-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia, Montigny, France) as described previously.23 Mouse monoclonal IgG2a antibody T101 and its fragment T101-F(ab)2 react with the CD5 surface antigen.23 An average of two ricin A-chain molecules were linked per antibody or Fab fragment. The amount of RTA-ITs, which contained no detectable ricin B-chain, is expressed as molar concentrations of bound A-chain.

Drugs and reagents. CsA and SDZ PSC 833 were kindly supplied by Sandoz (Basel, Switzerland) and Pharmaceutical Inc (East
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 non-specific 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. 1 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 10^5 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%.

125I labeling of T101 RTA-IT. T101 RTA-IT (50 μg) was incubated with 1 mCi of 125I (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 125I 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 Briefly, CEM III cells were incubated for 1 hour at 4°C with 125I 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^-8 mol/L) 2 hours before addition of 125I-T101 RTA-IT. After three ice-cold washes with saline solution (removing excess 125I-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 125I-ligand, was determined by radioactivity counting. 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).

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 CsA, 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)_2 RTA-IT cytotoxicity on CEM III cells 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 \(\mu\)mol/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).

**Effect of Cs on the rate of protein-synthesis inhibition by anti-CD5 RTA-ITS.**  The inhibition by T101 RTA-IT (1 \(\times\) \(10^{-8}\) mol/L) of protein synthesis reached approximately 50% after 24 hours, whereas in the presence of 4 \(\mu\)mol/L CsA and 4 \(\mu\)mol/L SDZ PSC 833 it increased to 90% and 94%, respectively (\(P < 0.025\)) (Fig 2A). Compared with its whole-fragment counterpart, T101 F(ab')\(_2\) RTA-IT, reached 94% inhibition of protein synthesis within 24 hours, which was further increased to almost 99% with both 4 \(\mu\)mol/L CsA and 4 \(\mu\)mol/L SDZ PSC 833 (\(P < 0.05\)) (Fig 2B).

**Enhancement of T101 RTA-IT in human plasma.** 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 \(\times\) \(10^{-8}\) 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 infusion, T101 RTA-IT cytotoxicity increased to 61%, 77%, 85%, and 77%, respectively.

**Effects of CsA on internalization and degradation of \(^{125}\)I-T101 RTA-IT.** CEM III cells were incubated with a membrane-saturating concentration of \(^{125}\)I-T101 RTA-IT (1 \(\times\) \(10^{-8}\) mol/L) for 1 hour at 4°C. After washing in ice-cold saline solution (removing excess \(^{125}\)I-RTA-IT), CEM III cells were incubated at 37°C with or without 4 \(\mu\)mol/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 \(\mu\)mol/L CsA did not significantly modify the kinetics of internalization.

After protease treatment, CEM III cells were lysed and intracellular nondegraded and degraded \(^{125}\)I-ligand was

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

<table>
<thead>
<tr>
<th>RTA-IT</th>
<th>Drug</th>
<th>(\mu)mol/L</th>
<th>(IC_{50}) ((\mu)mol/L)</th>
<th>Fold Enhancement</th>
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<tr>
<td>T101</td>
<td>No drug</td>
<td>25,000 ± 5,000</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CsA</td>
<td>1.0 18,000 ± 2,000</td>
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<td>2.0 1,233 ± 2,516</td>
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<td>4.0 247 ± 50</td>
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</tr>
<tr>
<td></td>
<td>PSC 833</td>
<td>1.0 19,333 ± 1,154</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 866 ± 1,527</td>
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<tr>
<td></td>
<td></td>
<td>4.0 237 ± 55</td>
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<tr>
<td>T101 F(ab')(_2)</td>
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<td>2,366 ± 321</td>
<td>1.3</td>
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<tr>
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<td>CsA</td>
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<td>1.3</td>
<td></td>
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<td>4.0 253 ± 25</td>
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<td>2.0 933 ± 153</td>
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<td></td>
<td></td>
<td>4.0 290 ± 36</td>
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<td></td>
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<tr>
<td>Ricin A-chain</td>
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<td>273,333 ± 25,166</td>
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<td>PSC 833</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 \(IC_{50}\) values were determined. Values are mean of three independent experiments ± SD.
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Evaluating 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-

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Fig 2. Effect of CsA and PSC 833 on the kinetics of protein-synthesis inhibition by anti-CD5 RTA-ITs. CEM III cells were incubated with 1 × 10^-8 mol/L T101 RTA-IT (A) or T101 F(ab)2 RTA-IT (B) in the absence (□, r = .88 and .98, respectively) or in the presence of 4 μmol/L CsA (■, r = .99 and .95) and 4 μmol/L PSC 833 (○, r = .99 and .96) for the indicated times and then assayed for protein synthesis. Points are the means of triplicate determinations with SD no greater than 10%.

Fig 3. Cytotoxicity of T101 RTA-IT in human plasma on CEM III cells. CEM III cells (2 × 10^6) were incubated for 18 hours with either T101 RTA-IT or ricin A-chain in the presence of 87% human plasma obtained from four patients before (■) and 2 hours after CsA infusion (○). Protein synthesis was then measured. Points are the means of triplicate determinations.

Fig 4. Internalization rate of 125I-T101 RTA-IT in CEM III cells. CEMIII cells were incubated with 125I-T101 RTA-IT (1 × 10^-8 mol/L) in the absence (□, r = .99) or in the presence of 4 μmol/L CsA (■, r = .99) for the indicated times, at the end of which cells were washed. Internalized RTA-IT was measured as described in Materials and Methods. Points are the means of triplicate determinations with SD no greater than 10%.
were incubated for 4 hours at 37°C with 1 × 10^{-8} \text{mol/L } ^{125}\text{I}-

T101 RTA-IT with or without 4 \mu\text{mol/L CsA or } 4 \mu\text{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}\text{I}-T101 RTA-IT and 4 \mu\text{mol/L CsA or } 4 \mu\text{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. 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. 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\text{mol/L CsA and } 4 \mu\text{mol/L SDZ PSC 833 in vitro. The F(ab')2 fragment counterpart of the whole T101 Ig RTA-IT, although more potent, 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.

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. However, lysosomotropic amines, such as NH₄Cl, and carboxylic ionophores, such as monensin, do not alter either degradation or intracellular trafficking of internalized RTA-ITs. These agents do increase the kinetics of cytotoxicity.

T101 RTA-IT has a distinctive slow rate of cell intoxication and enhancers such as monensin and NH₄Cl are able to increase the rate of cell kill dramatically. 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 T101 and T101 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 T101 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. However, CsA has been speculated as possibly binding to lipid domains and thereby provoking changes in both membrane polarity and lipid order.

Among the limitations of RTA-IT therapy, CLS appears to be the most important side effect. The recent report
that CLS substantially increased in patients who also received conventional CsA immunophrophylaxis may limit their combined use.21 However, CLS can be treated by glucocorticoids,24 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,24 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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REFERENCES

21. Przepiorka D, LeMaistre CF, Luna M, Champlin RE: Cyclosporine (CsA) enhances the toxicity of anti-CD5 ricin A chain immunotoxin when used to prevent acute graft-versus-host disease (AGVHD) after allogeneic marrow transplantation (BMT). Blood 78:230, 1991 (abstr, suppl 1)

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Cyclosporin A and cyclosporin SDZ PSC 833 enhance anti-CD5 ricin A-chain immunotoxins in human leukemic T cells

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