Autocrine Growth of a Human T-Cell Line Is Inhibited by Cyclosporin A

By Alice Dautry-Varsat, Agnès Hémard, Véronique Cornet, and Véronique Duprez

The effect of cyclosporin A (CsA), a potent immunosuppressive agent, on a human T-cell line, IARC 301, which constitutively secretes interleukin-2 (IL-2) and expresses high-affinity IL-2 receptors, was investigated. We show that CsA inhibits IARC 301 cell growth. CsA also prevents the constitutive secretion of IL-2 in this T-cell line by blocking transcription of the IL-2 gene. If exogenous IL-2 is added together with CsA for 3 days, the cells grow as well as untreated controls. Thus, under such conditions, CsA inhibits IARC 301 growth by preventing its endogenous constitutive IL-2 synthesis. This demonstrates that IL-2 stimulates the proliferation of this cell line by an autocrine pathway, in agreement with our previous data. We also show for the first time, that CsA not only can inhibit IL-2 production of T cells upon activation, but that it can also prevent ongoing constitutive IL-2 synthesis of a T-cell line.

MATERIALS AND METHODS

Cells. The human cell line, IARC 301, was derived from the lymph nodes of a patient with a T lymphoma. The culture was established in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS) in the presence of feeder cells, irradiated human fibroblasts. It was then grown in the same medium, without feeder cells, for several months. This cell line is CD2+ , CD3+ , CD7+ , CD1+ , CD3+ , CD4+ , CD8+ , CD20+ , and Fc receptor and surface immunoglobulin negative. We tested for retroviral sequences by Southern blot analysis and could not detect HTLV I or HTLV II sequences in IARC 301 with HTLV I and II probes. No reverse transcriptase activity could be detected (data not shown).

For long-term culture, IARC 301 cell concentration was maintained between 2 x 10^5 and 1.3 x 10^6 cells/mL. IARC 301 cells were consistently shown to be free of mycoplasma. The human acute lymphocytic leukemia T-cell JURKAT was cultured in RPMI 1640, 10% FCS, 2 mmol/L L-glutamine supplemented with 10 mmol/L Hepes pH 7.3 and 5 x 10^-5 mol/L 2-mercaptoethanol.

Materials. Recombinant human IL-2 (Amersham, England) was used as the source of purified IL-2. Phytohemagglutinin M (PHA) was purchased from GIBCO (Grand Island, NY). CsA was a gift from Dr J.F. Borel (Sandoz, Switzerland). A stock solution at 10 mg/mL in 80% ethanol was prepared, and CsA was directly diluted from this stock solution into the culture medium at the indicated concentrations. Mononclonal anti-IL-2 receptor antibodies 18E6.4 and 39C6.52 were a gift from D. Olive, Marseilles, France. These antibodies inhibit IL-2-induced T-cell proliferation and recognize the 55-kd chain of the IL-2 receptor.

Acid pH elution of cell surface-bound IL-2. IL-2 was eluted from IARC 301 cell surface as described previously, and IL-2 bioactivity was determined by proliferation of the murine IL-2-dependent CTLL-2 line (subcloned in our laboratory, clone G4) by 1 U IL-2 is equal to 0.077 ng. In every IL-2 bioassay, a standard solution of known concentration, obtained from the Biological Response Modifiers Program of the National Cancer Institute (Bethesda, MD), was titrated as a control.

IARC 301 proliferation and growth assays. Cell growth was followed by measuring H-thymidine incorporation by counting live cells by trypan blue exclusion as described previously. When indicated, IL-2 or CsA were added at the beginning of the culture.

IL-2 mRNA analysis. Total cellular RNA was prepared by a guanidine isothiocyanate technique from exponentially growing IARC 301 cells and from cells incubated for 24 hours with CsA (0.1 or 0.5 μg/mL). Total cellular RNA was also prepared from exponentially growing JURKAT cells and from JURKAT cells incubated at 2 x 10^6/mL for 6 hours with 1% PHA and 10 ng/mL TPA. RNA samples were electrophoresed through 1.2% agarose gels containing formaldehyde. Transfer on uncharged nylon membrane.
(Amersham hybond N) was performed in 150 mmol/L ammonium acetate. RNA probes labeled with \(^{32}\)P-UTP at a specific activity of \(1.5 \times 10^7\) cpm/\(\mu\)g were obtained by in vitro runoff transcription using T3 RNA polymerase. Hybridization was performed at 55°C in a buffer containing 50% deionized formamide, 750 mmol/L NaCl, 150 mmol/L Tris, pH 8.0, 200 mmol/L sodium phosphate buffer, pH 6.8, 10 mmol/L EDTA, 0.1% sodium dodecyl sulfate (SDS), and 500 \(\mu\)g/mL heparin. Washing was done for 45 minutes at 68°C in 90 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 7.0, 0.5 mmol/L EDTA, 0.1% SDS, and 0.1% sodium pyrophosphate.

The plasmid used to transcribe the IL-2 probe, bs IL-2, was constructed by subcloning the 450 base pair (bp) Hinfl fragment of IL-2 cDNA in the bluestrip M13 vector (Vector Cloning Systems, San Diego). The Hinfl 1 fragment from plasmid pH-IL-2, a gift of Dr W. Fiers, was purified by electrophoresis, converted to a blunt-ends fragment using the Klenow fragment of Escherichia coli DNA polymerase I as described, and ligated to the bluestrip vector at the SmaI site with T4 DNA ligase.

**RESULTS**

CsA inhibits IARC 301 cell growth. We previously showed that the human tumor T cells IARC 301 secrete IL-2 and express IL-2 receptors on their surface without activation. Because of the known effects of CsA on T cells, we studied the effect of CsA on IARC 301 proliferation. CsA was added to IARC 301 cultures at concentrations varying between 0.01 and 0.5 \(\mu\)g/mL. \(^{3}H\)-Thymidine incorporation was measured 2 days later (Table 1), and cell growth was followed for 3 days with CsA by counting live cells (Fig 1A).

Both experiments showed that CsA inhibits IARC 301 growth. After 3 days in the presence of CsA, the cultures contained mostly dead cells and debris. Because CsA was dissolved in ethanol, parallel cultures were performed with ethanol at the concentrations used (between 1/2,000 and 1/100,000), ethanol slightly affects cell growth. As a control, we showed that CsA did not affect the growth of another human T cell line, JURKAT (data not shown and ref 10).

We tested the effect of IL-2 on the inhibition of growth caused by CsA. As shown in Table 1 and Fig 1B, IARC 301 cultured for 3 days with CsA (up to 0.5 \(\mu\)g/mL) and 10 U/mL recombinant IL-2 (Fig 1): indeed, we previously showed that at low cell densities, the concentration of secreted IL-2 is limiting for IARC 301 growth and these cells grow faster when IL-2 is added exogenously.

**Table 1. Proliferation of IARC 301 Cells With CsA and With or Without IL-2**

<table>
<thead>
<tr>
<th>Incubation With</th>
<th>(^{3}H)-Thymidine Incorporation</th>
<th>(%) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA 0.5 (\mu)g/mL</td>
<td>43,000</td>
<td>--</td>
</tr>
<tr>
<td>IL-2*</td>
<td>2,390</td>
<td>5.5</td>
</tr>
<tr>
<td>CsA 0.5 (\mu)g/mL + IL-2</td>
<td>89,000</td>
<td>--</td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA 0.01 (\mu)g/mL</td>
<td>66,700</td>
<td>--</td>
</tr>
<tr>
<td>IL-2*</td>
<td>102,000</td>
<td>66</td>
</tr>
<tr>
<td>CsA 0.01 (\mu)g/mL + IL-2</td>
<td>88,900</td>
<td>87</td>
</tr>
</tbody>
</table>

Cells were seeded at 2.5 \(\times\) \(10^6\) cells/mL in 0.2 mL culture medium with or without CsA or/dil 10 U/mL IL-2 and incubated for 45 hours. \(^{3}H\)-Thymidine (0.5 \(\mu\)Ci/0.2 mL) was added to the culture for the last 4 hours of incubation. Because CsA was dissolved in ethanol, ethanol was added to all the cultures at the same final concentration (a: 1/2,000; b: 1/100,000).

U/mL recombinant IL-2 grew as well as control untreated cells. Without CsA, IARC 301 cells grew faster with 10 U/mL IL-2 (Fig 1): indeed, we previously showed that at low cell densities, the concentration of secreted IL-2 is limiting for IARC 301 growth and these cells grow faster when IL-2 is added exogenously.

**Effect of CsA on IL-2 secretion by IARC 301 cells.** IARC 301 cells constitutively produce IL-2, and ~10% to 20% of the cell surface high-affinity receptors of exponentially growing cells bear IL-2. Treating cells at acidic pH (below 4) removes IL-2 from these receptors. This method previously allowed us to show that IL-2 can be eluted from the surface of exponentially growing IARC 301 cells. We studied the effect of CsA on IL-2 production in this way. IARC 301 cells (5 \(\times\) \(10^5\)/mL) were incubated for 6 hours with 0.5 \(\mu\)g/mL CsA, washed, and incubated for another 24
hours with 0.5 μg/mL CsA. After treatment with acid pH, <0.2 U IL-2 was eluted from the surface of 10^6 CsA-treated cells, whereas >30 U IL-2 could be eluted from the surface of 10^6 untreated cells (Fig 2A). Thus, at least 150-fold less IL-2 is found on the receptors of cells treated with 0.5 μg/mL CsA than on untreated cells.

Although IARC 301 cells secrete IL-2, IL-2 concentration in the culture medium is low because IL-2 is constantly internalized into the cells and degraded after binding to surface high-affinity receptors. To prevent consumption by IARC 301 cells of the IL-2 they secrete, we used an anti-IL-2 receptor antibody. We assumed that an antibody that inhibits proliferation of IL-2--dependent cell lines might do so by competing with IL-2 for binding to the receptors. Such an antibody is expected to prevent binding and consequent degradation of IL-2 secreted by IARC 301 cells. Such was the case, as shown in Fig 2B: When 18E6.4 monoclonal antibody was added to the culture for 24 hours, the concentration of secreted IL-2 in the medium was ~1 U/mL. This represented ~30-fold more IL-2 than that found in the culture medium of untreated exponentially growing cells. 20 When the cells were incubated with 0.5 μg/mL CsA in addition to 18E6.4 antibodies, no IL-2 could be detected 40 hours later (Fig 2B). Similar results were obtained with monoclonal 39C6.5 (data not shown). Thus, as measured in the presence of anti-IL-2 receptor antibodies, CsA decreased IL-2 production at least 60-fold.

CsA inhibits accumulation of IL-2 mRNA in IARC 301 cells. To determine whether the lack of IL-2 secretion by IARC 301 cells in the presence of CsA was owing to a lack of mRNA coding for this lymphokine, we performed Northern blot analysis. RNA was prepared from exponentially growing IARC 301 cells and from cells treated with 0.1 and 0.5 μg/mL CsA for 24 hours, size-fractionated on formaldehyde agarose gels, and analyzed by RNA transfer blot hybridization with a probe for IL-2, bsIL-2. As shown in Fig 3, IL-2 mRNA was detected in untreated cells and CsA decreased IL-2 mRNA production.

**DISCUSSION**

CsA primarily affects T cells, and its immunosuppressive effect results from interference with T cell functions. Exactly where CsA exerts its effects on T lymphocyte function is unclear, but its inhibition of IL-2 production by stimulated T cells is well documented. 3,9,13 We previously showed that IARC 301, a human tumor T cell line, spontaneously secretes IL-2 and constitutively expresses high-affinity IL-2 receptors. 20 In the present study, we showed that CsA inhibits IL-2--dependent growth of this
cell line. Under conditions in which CsA kills IARC 301 cells, it does not affect proliferation of JURKAT T cells whose growth is IL-2 independent. Neither the IL-2 mRNA nor the lymphokine itself (in the culture supernatant or bound to cell surface receptors) could be detected after a 24-hour incubation with 0.5 μg/mL CsA. The growth inhibition effect of CsA on IARC 301 could be reversed by simultaneous addition of exogenous IL-2 at a concentration sufficient to saturate high-affinity IL-2 receptors. CsA inhibits only IL-2 synthesis in IARC 301 and not the constitutive expression of high-affinity surface IL-2 receptors, which are responsible for IL-2 effect on lymphocyte26 and IARC 301 proliferation.36 Indeed, the number of high-affinity IL-2 receptors was not decreased 16 hours after CsA addition35 or 24 or 48 hours after CsA addition (not shown); if anything, it was slightly increased.25 Together, these data show that CsA affects IARC 301 cell viability primarily by inhibiting its constitutive IL-2 synthesis and that IL-2 is an absolute requirement for IARC 301 growth. Thus, IL-2 stimulates the autocrine growth of this tumor cell line. The data we report are in agreement with the ability of monoclonal antibodies against IL-2 and IL-2 receptors to inhibit IARC 301 proliferation.20 Autocrine growth stimulation by IL-2 has been proposed in many cases and demonstrated in a few cases using monoclonal antibodies: a T cell clone,27 HTLV-I-infected cells,28 and an IL-2 dependent cell line, CTLL-2, infected with a retroviral vector that leads to constitutive expression of IL-2.29

IL-2 secretion in normal T lymphocytes requires activation of the T cell either by an antigen and antigen-presenting cells or by a mitogen or by anti-T cell receptor antibodies, and nonactivated T lymphocytes do not secrete IL-2. In activated T cells and T-cell lines, CsA inhibits IL-2 mRNA accumulation.16,31 In the case of JURKAT cells activated by TPA and PHA, this inhibition is at the transcription level.10 For the first time, we showed that CsA is able to inhibit expression of the IL-2 gene in a T-cell line in which IL-2 secretion is constitutive and does not require exogenous activation. Thus, CsA not only blocks induced IL-2 production, but also stops ongoing IL-2 synthesis. Similarly, CsA inhibits IL-2 synthesis in several instances of T cells that must be activated to produce IL-2, at a time after stimulation when transcription of the IL-2 gene has already started.6,13,15 These data and ours differ from results reported by Kronke and colleagues,10 in which the effect of CsA on IL-2 synthesis was less striking if it was added when IL-2 gene transcription had already started.

The mechanism of IL-2 gene deregulation responsible for constitutive IL-2 secretion in IARC 301 has not yet been elucidated. IARC 301 cells secrete IL-2 without an activation signal and do not express the T3 antigen, a component of the T cell receptor, ruling out a direct interference of CsA with some event involving the T cell receptor. As we showed, IARC 301 and normal T lymphocytes share a step—in the activation pathway leading to IL-2 secretion—that is blocked by CsA and appears to be downstream from antigen or mitogen binding. In agreement with this interpretation, CsA could not block binding of an anticonvulstic antibody on a murine T cell clone but inhibited IL-2 synthesis induced by this antibody.5

Growth inhibition by CsA and its reversal by IL-2 may be a very simple and useful test to determine whether growth of a cell line, or cells from a patient, is controlled by an autocrine mechanism involving IL-2. Autocrine growth stimulation by cytokines other than IL-2 has also been described in several instances of leukemia and leukemic cell lines.30,34 CsA inhibits production of several lymphokines, IL-1, IL-2, IL-3, IFN-γ, TNF-α, and TNF-β, and others,12,19 although it does not inhibit synthesis of some of them, such as GM-CSF.35 Autocrine growth of leukemic cells is generally difficult to test since antibodies against the cytokine or receptor are required and may not be available. Use of CsA in short-term culture to test for cell growth inhibition, without or with cytokines added to reverse an eventual inhibition, is simple and may be useful to assess autocrine growth of leukemic cells and define the cytokine involved.

Whether the growth of some tumor cells is governed by an autocrine mechanism in vivo is still open to question. In several instances, after injection to nude mice of autocrine cells, obtained either from human tumors or after transfection with appropriate oncogenes, tumors developed29,36-40, in some cases, growth of the tumors was inhibited by injecting antibodies directed against the growth factor.36,38 This shows that autocrine cells can induce tumors. In favor of the existence of autocrine tumors in humans is the fact that cells from a patient with adult T-cell leukemia were shown to depend on IL-2 by an autocrine mechanism, as tested after short-term (1 to 3 days) culture in vitro.27

If indeed the growth of some lymphomas and leukemias in humans at certain stages is governed by an autocrine mechanism, in cases in which this can be assessed by short-term culture in vitro CsA treatment of patients with such tumors may be clinically valuable.

ACKNOWLEDGMENT

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