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Immunology

Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription

(*in vitro* transcription/lymphokine gene expression/interleukin 2)

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ABSTRACT **Cyclosporin A (CsA) is a potent immunosuppressive agent, now gaining wide application in human organ transplantation. The immunosuppressive activity of CsA is at least in part due to inhibition of lymphokine production by activated T lymphocytes. Specifically, inhibition of T-cell growth factor (TCGF; also designated interleukin 2) production appears to be an important pathway by which CsA impairs T-cell function. To define further both the specificity of CsA and the level at which it interferes with lymphokine gene expression, we have studied its effects on TCGF mRNA accumulation as well as TCGF gene transcription. These studies were performed with a cloned human leukemic T-cell line (Jurkat, subclone 32), which can be induced with phytohemagglutinin and phorbol 12-myristate 13-acetate to produce large amounts of TCGF. In these cells, high levels of TCGF mRNA were present in induced but not in uninduced Jurkat cells as judged by hybridization to a cloned human TCGF cDNA probe. CsA completely inhibited induced TCGF mRNA accumulation at concentrations of 0.3–1.0 μg/ml, whereas low levels of appropriately sized TCGF mRNA were present at 0.01 μg/ml. In nuclear transcription experiments, CsA inhibited the synthesis of TCGF transcripts in a dose-dependent manner with complete inhibition at a concentration of 1 μg/ml. In contrast, CsA did not inhibit the expression of two other inducible genes, TCGF receptor and HT-3. Further, HLA gene expression was also less affected than TCGF in CsA-treated cells. These data suggest a relatively selective action of CsA on TCGF gene transcription.**

Cyclosporin A (CsA), a fungal metabolite, has become a valuable drug in human organ transplantation, mainly because of its ability to prevent the rejection of HLA-mismatched allografts (1–3). Although CsA has no advantage over other immunosuppressive agents with respect to the frequency of post-transplant viral infections, it is nontoxic for bone marrow stem cells and results in fewer bacterial infections (1, 4).

It has been shown that CsA acts primarily on T lymphocytes sparing the immunocompetence of B cells and macrophages (5–8). CsA immunosuppression is mediated at least in part by inhibition of lymphokine secretion by T cells, which provide requisite growth and differentiation signals for T cells, B cells, and macrophages, (9–12). Among these lymphokines is T-cell growth factor (TCGF, or interleukin 2), the principal stimulus causing proliferation of activated T lymphocytes (13). CsA acts at least in part by interfering with the production of TCGF. However, the precise mechanism by which CsA impairs T-cell function in general and TCGF production in particular remains unresolved.

In an attempt to dissect the mechanism of CsA-mediated immunosuppression, we have studied TCGF gene expression in a cloned leukemic T-cell line (Jurkat, subclone 32). Using a cloned cDNA probe for human TCGF (14), we have studied TCGF gene expression and CsA action at the level of mRNA production and gene transcription in isolated nuclei. We present evidence that CsA inhibits TCGF gene expression at the level of transcription, indicating its nuclear site of action. The inhibition of TCGF gene activation seems to be a selective feature of CsA, since the expression of HLA genes and other inducible genes, including the receptor for TCGF, was less affected.

MATERIAL AND METHODS

Cell Lines. Jurkat leukemic T cells (subclone 32) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. For induction, culture medium with 3% fetal calf serum, phytohemagglutinin (PHA, 1 μg/ml), and phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) was added to cells at a density of 4×10^6 cells per ml.

Anti-Tac Binding Assay. Monoclonal anti-Tac was tritiated to high specific activity (13.6×10^6 dpm/μg) by reductive methylation as described (15). Briefly, Jurkat cells induced in the presence or absence of CsA were suspended in RPMI 1640 medium containing 1% bovine serum albumin, human IgG (1 mg/ml, Cutter), 25 mM Hepes, and 0.1% sodium azide. Aliquots of 10^6 cells were incubated in triplicate with 4.5 ng of ^{3}H-labeled anti-Tac (^{3}H-anti-Tac) in the presence of either control antibody, RPC-5 (100 μg/ml), or unlabeled anti-Tac (100 μg/ml) for 60 min at room temperature. Thereafter, cells were centrifuged in a Microfuge through 1 M sucrose cushions to separate free and cell surface-bound ^{3}H-anti-Tac. Radioactivity of the cell pellets was measured in a liquid scintillation counter and specific ^{3}H-anti-Tac binding was determined.

Cytoplasmic Dot Blot Hybridizations. Relative levels of specific mRNA species were determined by cytoplasmic dot blot hybridization as described by White and Bancroft (16). Following induction under varying conditions, 2×10^6 Jurkat cells were lysed in a buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.5% Nonidet P-40. Nuclei were pelleted, and the supernatants were denatured in 7.4% formaldehyde/0.9 M NaCl/0.09 M sodium citrate, pH 7, by heating at 60°C for 15 min. Samples were then diluted 1:2 in 2.25 M NaCl/225 mM sodium citrate, pH 7, and spotted onto nitrocellulose filters by using a manifold filter apparatus (Schleicher & Schuell).

RNA Transfer Blotting Analysis. Total cellular RNA was prepared by a guanidine isothiocyanate method (17), and poly(A)-RNA was selected by oligo(dT)-cellulose chromatography (18). Poly(A)-RNA was size fractionated on formaldehyde-agarose gels and transferred to nitrocellulose filters (19).

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Abbreviations: CsA, cyclosporin A; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; TCGF, T-cell growth factor.

***In Vitro* Labeling of RNA in Isolated Nuclei.** *In vitro* transcription with isolated nuclei was performed according to the method of McKnight and Palmiter (20). To isolate nuclei, Jurkat cells were washed in buffer containing 10 mM Tris (pH 7.5), 2 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM dithiothreitol, and 0.3 M sucrose. Cells were lysed in the same buffer supplemented with Nonidet P-40 at a final concentration of 0.02% and centrifuged in a Beckman SW 41 rotor at 25,000 rpm over a 2 M sucrose gradient to pellet the nuclei. For *in vitro* transcription, 4–5 $\times 10^7$ nuclei were incubated for 30 min at 26°C in a 200-μl reaction solution containing 100 mM KCl, 4.5 mM $MgCl_2$, 0.4 mM (each) ATP, CTP, and GTP, 2 mM dithiothreitol, 20% glycerol, and 0.2 mCi (1 Ci = 37 GBq) of [^{32}P]UTP (3000 mCi/mmol, Amersham). Incorporation of [^{32}P]UTP increased both with time of incubation and number of nuclei added. The reaction mix was then treated with DNase I (100 μg/ml) for 15 min at 26°C, followed by digestion with proteinase K at a concentration of 150 μg/ml. Nuclear RNA was then isolated by hot phenol/chloroform extraction and two cycles of ethanol precipitation.

cDNA Probes. The origin and characterization of the 1.1-kilobase cDNA for human TCGF has been described in detail by Clark *et al.* (14). Plasmid pBR322 DNA, containing complementary DNA specific for HLA-B7 (21), was a kind gift from S. M. Weissman (Yale University, New Haven, CT). The 1.4-kilobase insert was purified by *Pst* I digestion, preparative agarose-electrophoresis, and electroelution. HT-3 cDNA recognizes mRNA sequences that are expressed selectively in activated mature T cells and human T-cell lymphoma/leukemia virus-infected T-cell lines (22). The 1.37-kilobase HT-3 fragment, derived from a cDNA library of the HUT 102 leukemic T-cell line, was prepared as described (22). For hybridization, cDNA probes were nick-translated with [^{32}P]dCTP (3000 Ci/mmol) to specific activities of 2–5 $\times 10^8$ cpm/μg.

Hybridization of DNA and RNA. For hybridization of RNA blots to specific cDNA sequences, nitrocellulose filters were prehybridized in 40% formamide/0.6 M NaCl/0.06 M sodium citrate, pH 7/1× concentrated Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/salmon sperm DNA (100 μg/ml)/10% dextran sulfate for 4–16 hr at 40°C. Thereafter, filters were incubated with 2–5 $\times 10^6$ cpm of nick-translated probes per ml in the same buffer for 16 hr at 40°C. In the nuclear transcription assay, ^{32}P-labeled nuclear RNA was hybridized to an excess of specific cDNA immobilized on nitrocellulose filters in 40% formamide/0.6 M NaCl/0.06 M sodium citrate, pH 7/5 mM EDTA/0.4% $NaDodSO_4$/1× concentrated Denhardt's solution/yeast tRNA (100 μg/ml) for 72 hr at 40°C. To immobilize excess amounts of cDNA, 0.5 μg of specific sequences was denatured by boiling for 10 min and treatment with 1 M NaOH. Following neutralization, probes were subsequently spotted onto nitrocellulose filters by using a manifold apparatus from Schleicher & Schuell. Following hybridization, all filters were washed four times in 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% $NaDodSO_4$ at room temperature and then twice in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% $NaDodSO_4$ at 50°C. The filters were then exposed at −70°C to Kodak XAR film using intensifying screens. Where indicated, autoradiograms were analyzed with a Biomed SL 504 laser densitometer, and the radioactivity of nitrocellulose filters was counted in a liquid scintillation counter.

RESULTS

CsA Inhibits the Accumulation of TCGF mRNA in Induced Jurkat Cells. Previous studies have indicated that CsA inhibits TCGF production by antigen- or mitogen-activated T cells (9, 10, 12). However, these studies did not define the mechanism of CsA action. Employing a cloned cDNA probe for human TCGF, we first determined TCGF mRNA levels in cloned leukemic T cells (Jurkat, subclone 32), either uninduced or induced with PHA (1 μg/ml) and PMA (50 ng/ml) for 6 hr in the presence or absence of varying concentrations of CsA. Poly(A)-RNA was then isolated from each cell population, size fractionated on formaldehyde-agarose gels, and analyzed by RNA transfer blot hybridization with nick-translated cDNA for TCGF. As shown in Fig. 1, TCGF mRNA was readily detected as an 11–12S species in induced Jurkat cells (lane 2), whereas no TCGF mRNA was detected in uninduced cells (lane 1). CsA at concentrations of 1 μg/ml (lane 3) or 0.3 μg/ml (lane 4) completely inhibited TCGF mRNA production. As concentrations of CsA were decreased from 0.1 to 0.01 μg/ml, increasing amounts of appropriately sized TCGF mRNA were identified (lanes 5–7). As shown in lane 8, the solvent for CsA, ethanol, did not interfere with TCGF mRNA production when added at a concentration of 0.1%, which was required to prepare a final CsA concentration of 1 μg/ml. Also seen are higher molecular weight mRNA species hybridizing to the TCGF probe. These mRNAs were absent in samples from uninduced and CsA-treated cells. By using an intron probe prepared from a TCGF genomic clone (provided by G. R. Crabtree), each of these larger mRNAs hybridized while the mature TCGF mRNA did not, suggesting that each represented a precursor form of TCGF mRNA. It should be noted that in some experiments of similar design, low levels of TCGF mRNA were detected at CsA concentrations of 1.0 μg/ml.

Selectivity of CsA. CsA did not diminish cell viability or the overall synthesis of DNA, RNA, or proteins, in either unstimulated or induced Jurkat cells (data not shown). Thus, CsA did not appear to exert a general inhibitory action on cell metabolism. To further examine the possibility of selectivity of CsA inhibition, we investigated its effects on the expression of membrane receptors for TCGF. Using a monoclonal anti-human TCGF receptor antibody, anti-Tac (23, 24), we have recently demonstrated that Jurkat cells express TCGF receptors following induction with PHA and PMA (25). The results summarized in Table 1 demonstrate that

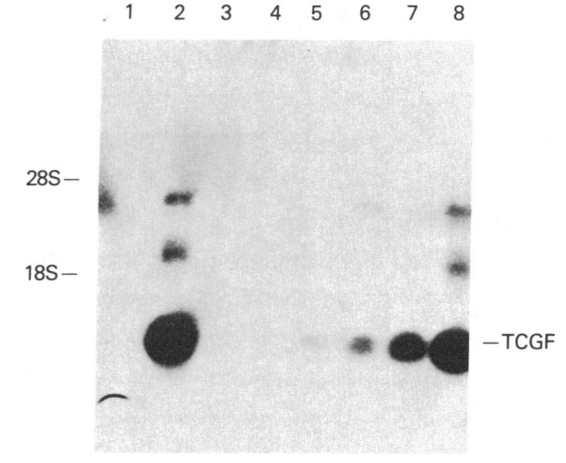

FIG. 1. Dose-dependent inhibition of TCGF mRNA accumulation by CsA. Poly(A)-RNAs from Jurkat cells were electrophoresed, transferred to nitrocellulose filters, and subsequently hybridized to nick-translated TCGF cDNA. Samples were 5 μg of poly(A)-RNA (each) of uninduced Jurkat cells (lane 1), induced with PHA (1 μg/ml) and PMA (50 ng/ml) for 6 hr (lane 2), or induced in the presence of CsA at concentrations of 1.0 μg/ml, 0.3 μg/ml, 0.1 μg/ml, 0.03 μg/ml, and 0.01 μg/ml (lanes 3–7, respectively). Lane 8 represents induction in the presence of 0.1% ethanol. The migration of 28S and 18S rRNA was determined by ethidium bromide staining.

Table 1. CsA does not inhibit PHA- and PMA-induced expression of TCGF receptors in Jurkat cells

Jurkat cells induced with	^{3}H-anti-Tac bound, pg per 10^6 cells
Medium alone	0
PHA and PMA	1114
+CsA, 1 μg/ml	
4 hr prior to induction	1506
1 hr prior to induction	1701
Simultaneously	1414
+CsA, 0.3 μg/ml	
Simultaneously	1787

Jurkat leukemic T cells were cultured for 20 hr in RPMI 1640 medium. 10% fetal calf serum with and without PHA (1 μg/ml) and PMA (50 ng/ml) and in the presence or absence of CsA. CsA was added at indicated concentrations either simultaneously with inducing agents or 1 or 4 hr prior to induction. Induced expression of TCGF receptors was measured in a radioreceptor binding assay by using ^{3}H-anti-Tac.

CsA at concentrations of 1.0 μg/ml and 0.3 μg/ml does not inhibit TCGF receptor expression in induced cells as measured by ^{3}H-anti-Tac binding. Furthermore, preincubation of cells with CsA for 4 or 1 hr prior to induction had no effect on ^{3}H-anti-Tac binding. In addition to TCGF receptor expression, we evaluated CsA effects on mRNA levels for HLA-B, constitutively expressed in Jurkat cells, and HT-3, a gene activated in Jurkat cells by PHA and PMA (22). Poly(A)-RNA from uninduced, induced, and CsA-treated cells was hybridized to nick-translated cloned cDNA probes specific for either TCGF, HLA-B7, or HT-3 (Fig. 2). As expected, induced but not uninduced Jurkat cells expressed TCGF mRNA, and this induced expression was inhibited by CsA (lanes 1–3). In contrast, similar levels of HLA mRNA were detected in uninduced and induced Jurkat cells (lanes 7 and 8). CsA only marginally decreased HLA mRNA levels (lane 9). HT-3 mRNA was not detected in uninduced Jurkat cells (lane 4). Upon induction, Jurkat cells produced HT-3 mRNA (lane 5), which was not inhibited by CsA at a concentration of 1 μg/ml (lane 6).

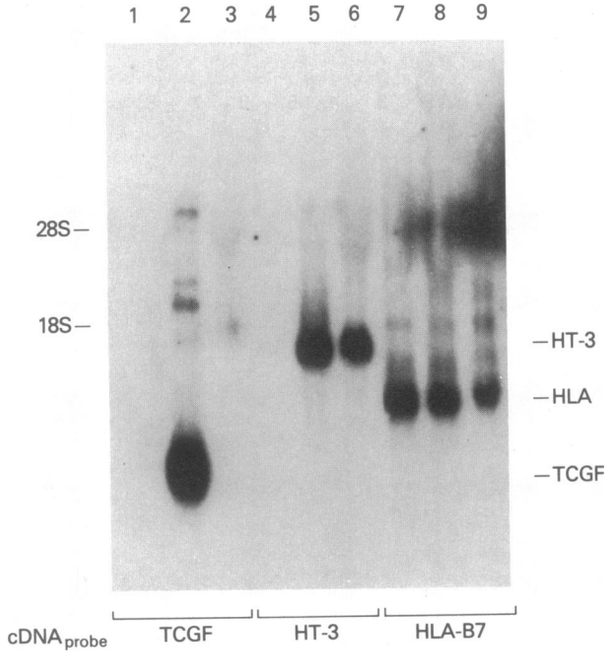

FIG. 2. Selectivity of CsA. Poly(A)-RNAs from Jurkat cells either uninduced (lanes 1, 4, and 7), induced (lanes 2, 5, and 8), or induced in the presence of CsA at 1.0 μg/ml (lanes 3, 6, and 9) were size fractionated and hybridized to [^{32}P]cDNA probes specific for TCGF (lanes 1–3), HT-3 (lanes 4–6), and HLA-B7 (lanes 7–9). The migration of 28S and 18S rRNA is indicated.

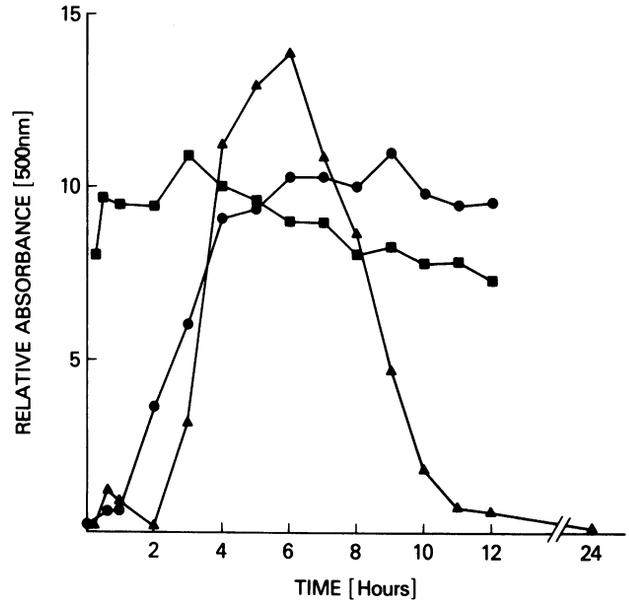

FIG. 3. Time course of TCGF, HLA, and HT-3 mRNA accumulation. Jurkat cells (2×10^6) were induced with PHA (1 μg/ml) and PMA (50 ng/ml). At the indicated times, cells were harvested, and cytoplasmic extracts were then spotted in serial 1:2 dilutions onto nitrocellulose filters and hybridized to either TCGF (▲), HLA-B7 (■), or HT-3 (●) cDNA probes. Autoradiograms were quantitated by densitometer reading and relative mRNA levels are expressed as percentage of the overall specific mRNA accumulation integrated for the time period investigated.

However, these results did not exclude the possibility of a rapid induction of HT-3 mRNA production prior to achieving effective intracellular levels of CsA. To rule out this possibility, we performed time course studies of TCGF, HT-3, and HLA mRNA accumulation employing cytoplasmic dot blot hybridizations. As shown in Fig. 3, the time course of induction was similar for both TCGF and HT-3. TCGF mRNA production was detectable at 3 hr, peaked at 6 hr, and declined to undetectable levels at 24 hr, confirming the results from a previous report (14). HT-3 mRNA accumulation started at 1 hr, peaked at 4 hr, and remained at this level for several hours. In contrast, HLA mRNA was constitutively expressed in unstimulated cells, slightly elevated at 3 hr after induction, and slowly declined over the next 9 hr.

We also determined the effects of CsA on TCGF and HT-3 mRNA levels when administered prior to, simultaneous with, or after induction with PHA and PMA. As shown in Fig. 4, 1-hr preincubation or concurrent administration of CsA resulted in almost complete inhibition of TCGF mRNA production. Thereafter, increasing amounts of mRNA levels emerged. When added 4 hr after induction, CsA did not alter TCGF mRNA levels. In contrast, CsA had no effect on HT-3 mRNA expression when added 1 hr prior to induction or at any time thereafter.

CsA Inhibits TCGF Gene Transcription. Although low levels of TCGF mRNA in CsA-treated cells were consistent with diminished TCGF gene transcription, these data did not exclude altered RNA splicing, processing, or transport combined with intranuclear RNA degradation or accelerated cytoplasmic mRNA degradation. To evaluate potential CsA effects on the transcription of TCGF-specific sequences directly, nuclei from uninduced Jurkat cells as well as from Jurkat cells induced in the presence or absence of CsA were isolated, and nascent RNA chains were allowed to elongate

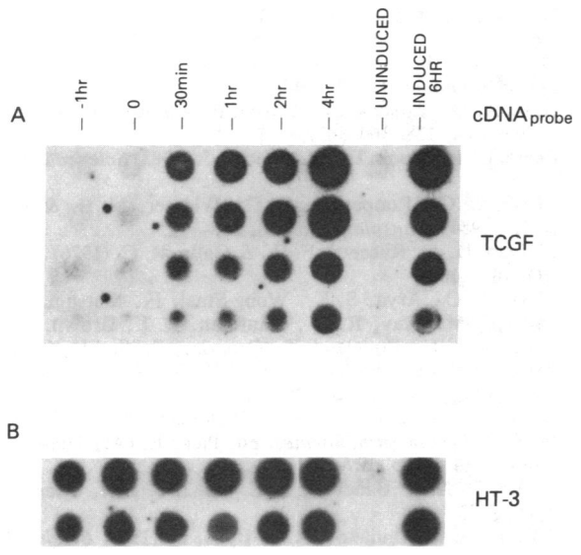

FIG. 4. Time course of CsA action. Jurkat cells were induced as described in the legend to Fig. 3 and, at the indicated times, CsA was added to a final concentration of 1 μg/ml. Serial 1:2 dilutions of cytoplasmic RNA were then spotted onto nitrocellulose filters and hybridized to either TCGF (*A*) or HT-3 (*B*) cDNA probes.

for 30 min in the presence of [^{32}P]UTP. Equivalent amounts of labeled nuclear RNA from each sample were then hybridized to excess cDNA specific for TCGF or HLA. A typical experiment is depicted in Fig. 5 and results analyzed quantitatively are given in Table 2. Significant levels of TCGF transcripts were observed in induced cells but not in uninduced cells. At a concentration of 1 μg/ml, CsA reduced TCGF transcription in nuclei from induced cells to background levels. In nuclei from cells treated with 0.1 and 0.01 μg of CsA per ml, TCGF transcription was suppressed by ≈78% and ≈67%, respectively. As expected, HLA sequences were found to be constitutively transcribed. However, in induced cells, HLA was transcribed at a 4- to 5-fold higher rate, as determined by both densitometry and counting. At 1 μg of CsA per ml, the transcription rate of HLA was intermediate between induced and uninduced cells. With decreasing concentrations of CsA, HLA transcriptional activity increased to levels observed in induced cells. As expected, nuclei from an Epstein–Barr virus-transformed B-cell line produced HLA- but not TCGF-specific transcripts. When nuclei were incubated in the presence of α-amanitin (5 μg/ml), [^{32}P]UTP incorporation was only 30–40% of untreated control, whereas the transcription of both TCGF and HLA were inhibited completely (data not shown), indicating that the transcription of both genes required intact RNA polymerase II activity.

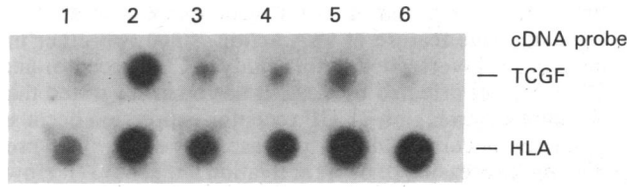

FIG. 5. CsA inhibition of TCGF gene transcription in isolated nuclei. Nuclei were isolated from unstimulated Jurkat cells (lane 1), from Jurkat cells induced for 4 hr in the absence (lane 2) or presence of CsA (1.0 μg/ml, 0.1 μg/ml, 0.01 μg/ml, lanes 3–5, respectively), and from an Epstein–Barr virus-transformed B-cell line (lane 6). Following *in vitro* transcription, RNA isolation, and hybridization to TCGF and HLA-B7 cDNA immobilized on nitrocellulose filters, hybridized ^{32}P-labeled nuclear RNA was then visualized by autoradiography.

DISCUSSION

Using a cDNA probe specific for human TCGF, we have employed RNA transfer blot analysis and *in vitro* transcriptional assays with isolated nuclei to study the effects of CsA on TCGF gene expression. We demonstrate that induction of Jurkat leukemic T cells with PHA and PMA leads to increased transcriptional activity of the TCGF gene, whereas in uninduced Jurkat cells TCGF-specific transcripts were not identified. Thus, the TCGF mRNA accumulation observed in induced cells reflects increased transcriptional activity of the TCGF gene. Similar, though slower, kinetics of TCGF mRNA accumulation in PHA-activated human lymphocytes have been reported by using an oocyte translation system (26). As noted previously (14), Jurkat TCGF mRNA levels rapidly decreased after peaking at 6 hr, suggesting a decline rate of ≈3 hr. Since TCGF transcription continues for at least 15 hr following induction (unpublished data), these data suggest that nontranscriptional regulatory mechanisms are involved in the rapid fall of TCGF mRNA levels. Since these studies were performed with a cloned T-cell line, the data indicate that the accumulation of TCGF mRNA and its subsequent decline result from intracellular events and occur in the absence of positive or negative signals from a second cell type.

It was previously reported that CsA abrogates TCGF production by activated T lymphocytes (9, 10, 12). However, it was not known whether CsA acted at the level of gene transcription, mRNA translation, or post-translational protein processing. The results of our study demonstrate that TCGF mRNA accumulation in induced Jurkat cells is diminished by CsA in a dose-dependent manner and that CsA acted by blocking TCGF mRNA transcription. Although we cannot exclude additional effects of CsA on TCGF mRNA processing, stability, or translation, these data indicate that one site of CsA action is in the nucleus at the level of TCGF gene

Table 2. Transcription of TCGF and HLA RNA in isolated nuclei of Jurkat cells

Nuclei		Input of [^{32}P]RNA, cpm × 10^{-6}	Hybridization (cpm) to		Background hybridization, cpm*	Relative transcription, ppm†	
Lane	Source		TCGF	HLA		TCGF	HLA
1	Jurkat cells uninduced	2.3	74	138	76	0.0	26.8
2	Jurkat cells induced	2.3	258	362	78	76.9	121.4
3	+ CsA, 1.0 μg/ml	2.7	88	236	84	1.4	55.4
4	+ CsA, 0.1 μg/ml	2.1	104	239	68	16.7	78.9
5	+ CsA, 0.01 μg/ml	2.5	147	448	79	26.7	148.7
6	Epstein–Barr virus-transformed B-cell line	1.9	62	574	60	1.0	263.6

Radioactivity from blots shown in Fig. 5 was measured in a liquid scintillation counter. Lanes 1–6 correspond with lanes 1–6 in Fig. 5.
*Background hybridization was determined by hybridization to pBR322.
†Background hybridization was subtracted from actual cpm and divided by input cpm to give specific hybridization values (ppm).

transcription. Inhibition of TCGF gene expression appears to be a selective feature of CsA action, since two other inducible genes investigated in this study, TCGF receptor and HT-3, were not affected by CsA. It has been suggested that CsA might fail to inhibit TCGF receptor expression because this antigen occurs at the early phase of the cell cycle preceding the expression of other activation antigens (27). However, different kinetics of expression of HT-3 and TCGF receptors could not account for their resistance to CsA treatment since (*i*) HT-3 exhibited similar kinetics of appearance compared with TCGF and (*ii*) both HT-3 and TCGF receptors were expressed in cells pretreated with CsA either 4 or 1 hr prior to the addition of inducing agents.

In addition to TCGF, CsA has been shown to inhibit interferon-γ production (11) and the expression of T-cell activation antigens such as HLA-Dr determinants (27, 28), transferrin receptors (T9, ref. 27), and T10 (27). Interestingly, TCGF regulates the expression of transferrin receptors in T cells (29), and interferon-γ enhances the expression of HLA class I and class II antigens (30, 31). Thus, CsA inhibition of TCGF gene expression could result in diminished transferrin receptor display. Similarly, CsA inhibition of the increase in HLA expression occurring after induction may be secondary to inhibition of interferon-γ production. Taken together, it is possible that CsA directly affects the expression of only a few inducible genes, while the apparent inhibition of others is the result of diminished TCGF or interferon-γ production. We believe that CsA, in addition to its clinical utility in human organ transplantation, may be valuable in further studies of differential lymphokine gene expression at the molecular level.

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