Transcription of IL-2 and IL-4 Genes Is Not Inhibited by Cyclosporin A in Competent T Cells*

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Cyclosporin A (CsA),1 a biologically active fungal metabolite, has a selective immunosuppressive effect on T-cell function (1). In resting T cells, CsA inhibits the proliferation of T cells stimulated by most, but not all, mitogens, antibodies to cell surface receptors, or antigens. It is now established that CsA inhibits T-cell proliferation primarily by blocking the transcription of several early activation genes, especially those of the important T-cell growth factors IL-2 and IL-4. This inhibition by CsA appears to be indirect, through inhibition of the regulators of growth factor transcription. Thus, CsA is a potent inhibitor of the activity of NF-AT, a factor critical for IL-2 gene transcription (2, 3). Inhibition of NF-AT is relatively selective since other transcription factors including Oct-1, AP-1, AP-3, and NF-kB are much less affected (2). IL-4 has recently been shown to support T-cell proliferation through a pathway which is independent of IL-2/IL-2 receptor (IL-2R) interaction (4). Similar to IL-2, the transcription of IL-4 is also inhibited by CsA, and the IL-4 gene has a putative NF-AT binding site (3, 5).

We have investigated the differential effects of CsA on the initiation or progression phases of human T-cell proliferation (6, 7). Brief exposure (30 min) to the phorbol ester, phorbol 12,13-dibutyrate (PDBu), and the calcium ionophore, ionomycin, CsA no longer inhibits cell cycle progression supported by the presence of PDBu alone. Here it is shown that transcription of the IL-2 and IL-4 genes occurs normally throughout this “progression” phase, even in the presence of CsA. However, further production of functional NF-AT, which began during the competence phase of the cell cycle, is inhibited. These data indicate that, although the primary initiation of transcription of IL-2 and IL-4 mRNA during induction of competence may be NF-AT-dependent and CsA-sensitive, the augmentation in the progression phase is both NF-AT-independent and CsA-resistant.

MATERIALS AND METHODS

Medium and Reagents—RPMI 1640 medium was obtained from Gibco Laboratories. Fetal calf serum was obtained from HyClone and was present in all cultures at a concentration of 10% (v/v). Culture medium was supplemented with 2 mM glutamine (Gibco). Phorbol 12,13-dibutyrate (PDBu, Sigma) was dissolved at 10−3 M in dimethyl sulfoxide. Ionomycin (Calbiochem) was prepared as a 5 mM stock solution in dimethyl sulfoxide. Recombinant IL-2 and IL-4 were kindly provided through the Biological Response Modifier Program of the National Cancer Institute and Dr. P. Trotta, Schering-Plough Research Institute, Bloomfield, NJ, respectively. Cyclosporin A (CsA) (Sandoz) was prepared as a 1 mg/ml solution in ethanol. Dimethyl sulfoxide or ethanol at equivalent concentrations in culture were without effect on the responses studied.

Cells—Peripheral blood T cells isolated from healthy volunteers were used in all assays. Mononuclear cell suspensions were prepared by Ficoll-Hypaque gradient centrifugation, and T cells (E−) were obtained by E-rosette enrichment as described (8).

Analysis of DNA Synthesis—Thymidine incorporation was measured after a 6-h pulse with 5 μCi/ml of [3H]thymidine (6.7 Ci/mmol, ICN Biomedicals) by scintillation counting.

PCR-assisted mRNA Analysis—Total RNA was extracted from 1 × 10⁶ cells using a method described previously (9). The mRNA levels of interleukins were analyzed using the method of reverse transcription/polymerase chain reaction (RT/PCR) with either IL-2, IL-4, or β-actin specific primers in 50 μl of a reaction buffer containing 10 mM Tris/HC1 (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 μM concentration of each deoxynucleotide and 50 pm concentration of each oligonucleotide primer. IL-2, IL-4, or β-actin cDNA was generated from RNA by incubation with 2.5 units of Taq polymerase (Cetus) per reaction. Thirty cycles of amplification in the thermocycler (Perkin-Elmer Cetus Instruments) were used with cycle conditions of 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min. Ten μl of the PCR products were separated by gel electrophoresis in 8% polyacrylamide gels and stained with ethidium bromide.

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‡ The abbreviations used are: CsA, cyclosporin A; PDBu, phorbol 12,13-dibutyrate; RT/PCR, reverse transcription/polymerase chain reaction; bp, base pair(s); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I, ionomycin.

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visualized by staining with ethidium bromide. The oligonucleotide primers used were: IL-2, 5'-ACTCACCAGGTCT-ACAT (sense), 5'-AGGAATCATCAGGTCTTCAAG (antisense) (11); IL-4, 5'-CTT-CCCCCTCTGTCTTCTTCC (sense), 5'-TTCTCTGTCAGGCCTTT-TCAG (antisense) (5); β-actin, 5'-GAACTCCTCACTTCTTCATC (sense), 5'-GAGGATGATCAGACCTTCTAG (antisense) (12). The resultant PCR products were 267 bp (IL-2), 317 bp (IL-4), and 300 bp (β-actin). PCR products were desalted by dialysis against buffer C. Concentrations of proteins were determined as described previously (14), with modifications. Briefly, 5 × 10⁶ cells were washed once in ice-cold phosphate-buffered saline and subjected to centrifugation. The cell pellets were then washed once in 1 ml of buffer A (10 mM Hepes (pH 7.8), 15 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride), pelleted, and suspended in 1 ml of buffer B (buffer A plus 0.5% Nonidet P-40) for 10 min on ice. After centrifugation, the pelleted nuclei were suspended in 315 μl of buffer C (50 mM Hepes, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, 10% (v/v) glycerol, and 35 μl of S (NH₄)₂SO₄ (pH 7.9) was added. Following gentle mixing for 30 min, the resultant solution was vortexed at 200,000 × g for 15 min. An equal volume of 3 ml (NH₄)₂SO₄ was added to the supernatant, and precipitated proteins were pelleted at 100,000 × g for 10 min and suspended in 50 μl of buffer C. Protein samples were desalted by dialysis against buffer C. Concentrations of proteins were determined by Bradford assay (15). The binding reactions for NF-AT were performed with 10 μg of protein in a solution of 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 2 μg of poly[d(I-C)]. The protein solutions were incubated for 30 min at room temperature with 20,000 cpm of the [32P]-labeled double-stranded oligonucleotide (16). The samples were resolved on 4% polyacrylamide gels containing 2.5% glycerol in 0.25 mM Tris/HCl (pH 7.5), 10% glycerol, and 0.1× TBE buffer. The gels were then dried and bands were visualized by autoradiography. Confirmation that the band shifts being observed were due to binding of NF-AT was ascertained by competition assays using unlabeled (nonradioactive) oligonucleotides containing NF-AT binding sites, a technique described by others (16). Addition of the wild-type oligonucleotide (sequence listed above) eliminated the shifted band, whereas unrelated oligonucleotides, in 50-fold excess, failed to do so. Although identification of the retarded band as NF-AT utilized competition by a particular DNA sequence, as reported by others (2, 3, 16, 20), there may be limitations to this approach as it does not unequivocally identify a band in a gel.

RESULTS AND DISCUSSION

As shown in Fig. 1, either phorbol dibutyrate (PDBu, 10⁻⁸ M) or ionomycin (IL, 0.5 μM) alone did not induce any DNA synthesis in resting T cells. Treatment with both PDBu and ionomycin (PDBu/IL) for 48 h, induced a strong proliferative response in the T cells. CsA (1 μg/ml), when added from the initiation of the culture, completely inhibited DNA synthesis in T cells activated by PDBu/IL. After a brief exposure (30 min) to the combination of PDBu and ionomycin, T cells did not subsequently proliferate when these reagents were washed out (Fig. 1, PDB/I 30 min). This treatment did, however, render the cells "competent" in that they were induced to proliferate if PDBu, IL-2, or IL-4 was added in the progression phase. All of these stimuli, PDBu, IL-2, and IL-4, provide strong progression signals for competent T cells to progress to DNA synthesis. In these cases, the presence of CsA throughout the progression phase did not affect the proliferative response in competent T cells. Therefore, the immunosuppressive effects of CsA were clearly limited to the brief phase for induction of competence; once T cells became competent, CsA could no longer inhibit the progression of competent T cells to DNA synthesis triggered through at least two independent pathways, one IL-2-dependent, the other IL-4-dependent.

Since CsA is known to inhibit transcription of the IL-2 gene, we examined mRNA levels during the two phases of the proliferative response. In order to increase the sensitivity of mRNA detection, we used a PCR-assisted mRNA amplification method (10). First, we confirmed that PDBu/I-induced transcription of IL-2 was completely inhibited by CsA when the drug was added from the initiation of the culture (Fig. 2, A and B). On its own, PDBu was incapable of inducing IL-2 mRNA. Then we examined the effect of CsA on the transcription of IL-2 in the progression phase of competent cells. Fig. 3 illustrates the kinetics of IL-2 gene expression in competent cells following the 30-min incubation with and subsequent removal of PDBu/I. Following removal of the co-mitogens, some IL-2 mRNA was detected at 1 h in the absence of a progression signal; by 3 h, no IL-2 mRNA was detected. In parallel, no IL-2 protein was detected by bioassay (data not shown), nor was cell proliferation observed (Fig. 1). However, if PDBu was added to competent cells in the progression phase, levels of IL-2 mRNA were maintained at 3 h, increased by 6 h, and remained elevated for 12–24 h. This enhancement by PDBu in the progression phase was not affected by the presence of CsA at early time points in this phase of the culture. At later time points (12–24 h), mRNA levels were lower in CsA-treated cultures as shown in Fig. 1. However, these differences were not found to be statistically significant,
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Fig. 2. Inhibitory effect of CsA on the transcription of IL-2 in the initiation phase. A, T cells were cultured for 6 h under the conditions described, at which time total RNA was extracted. The mRNA levels of IL-2 or β-actin were analyzed using the method of RT/PCR described under "Materials and Methods." Ten μl of the RT/PCR products were subjected to electrophoresis in 8% polyacrylamide gel and visualized by staining with ethidium bromide. The resultant PCR product was 267 bp (IL-2) or 300 bp (β-actin). M above the first lane indicates DNA size markers (pBR322 plasmid digested with HaelIII). B, kinetics of IL-2 mRNA transcription. Total RNA was extracted at the indicated time and analyzed by RT/PCR followed by hybridization with a specific internal oligonucleotide probe as described under "Materials and Methods." The radioactivity of the band was measured by scintillation counting. Results of analysis of cells treated with PDBu/I are indicated by - - ; with PDBu/I + CsA by - - ; with PDBu alone by - - Δ. The results are representative of one of three similar experiments.

and levels of IL-2 protein were identical (~16 units/ml) when culture supernatants were harvested at 24 h for both CsA-treated and control cultures.

In many ways the production and role of IL-4 in T-cell growth parallel those of IL-2. IL-4 is responsible for up-regulation of its own receptor (17) and acts independently of IL-2 in triggering T-cell proliferation (18). Similar to IL-2 transcription, IL-4 expression is not induced by phorbol ester alone but only following co-incubation with ionomycin (5). Transcription of IL-4 is also inhibited when CsA is added from the initiation of culture, implying that similar transcriptional regulation may be involved in IL-2 and IL-4 gene expression. The effects of CsA added in the progression phase on IL-4 gene expression mimicked those for IL-2, although accumulation of the IL-4 message appeared to be lower than IL-2. Fig. 4 shows that addition of PDBu to competent cells in the progression phase enhanced the levels of IL-4 mRNA. Furthermore, the presence of CsA in the progression phase only minimally affected the induction of IL-4 mRNA by PDBu.

Fig. 3. CsA does not inhibit IL-2 transcription in the progression phase. T cells were first incubated with PDBu and ionomycin for 30 min, washed with medium three times, then incubated with medium alone, PDBu, or PDBu + CsA (1 μg/ml). Total RNA was extracted at the indicated times and analyzed by RT/PCR followed by hybridization with a specific internal oligonucleotide probe as described under "Materials and Methods." Ten μl of the RT/PCR products were loaded onto a nitrocellulose membrane using a slot-blot apparatus and hybridized with the internal oligonucleotide probe. The autoradiogram of one of three similar experiments is shown in panel A. The bands were then cut out of the membrane, and their radioactivity was measured by scintillation counting. Relative 32P incorporation to the control in each experiment (% control) was calculated; the control was established as 32P incorporation obtained in cells treated with PDBu/I for 30 min and then incubated in medium alone for another 30 min (12,124 ± 4736 cpm). The mean values (± S.D.) for three independent experiments illustrating the kinetics of IL-2 mRNA transcription are shown in panel B. Cells treated with PDBu/I for 30 min and then incubated in medium alone are indicated by - - ; with PDBu/I and then with PDBu by - - ; with PDBu/I and then with PDBu and CsA (1 μg/ml) by - - Δ. The p value between a and b by paired t test was 0.26.

These results indicate that once T-cell competence has been achieved, increases in levels of IL-2 or IL-4 mRNA can be induced by PDBu, which on its own is ineffective. This effect on gene transcription in the progression phase is refractory to the inhibitory effects of CsA observed when the drug is present from initiation of culture. Thus, the target for immunosuppression appears to lie within the initial 33-min stimulation of resting T cells. Since CsA causes a similar depolarization in resting and competent T cells (19), the results cannot be explained simply by the absence of any CsA effect on activated or competent cells (also see below). Among the earliest events which could serve as a target for CsA in the competence phase and possibly explain the resistance in the progression phase is activation of NF-AT as a regulator of cytokine gene expression. As discussed above, NF-AT serves as an essential regulatory factor in IL-2 and IL-4 gene transcription. CsA-mediated inhibition of NF-AT is apparently selective since other transcription factors are less affected (2). Activation of NF-AT was induced by the combination of PDBu/I and not by either drug alone, and
Fig. 4. CsA does not inhibit IL-4 transcription in the progression phase. The RNA preparations described in Fig. 3 were also analyzed for IL-4 mRNA using the same RT/PCR and slot blotting hybridization procedures described under "Materials and Methods." Panel A shows the autoradiogram of one of three similar blots, prepared as described in Fig. 3. Panel B shows the results (means ± S.D. of three separate experiments) of scintillation counting of the excised radioactive bands; that is, the kinetics of IL-4 mRNA synthesis. The control was determined as in Fig. 1dO 1.10

Fig. 5. Analysis of the cellular content of the transcription factor NF-AT by the electrophoretic mobility-shift assay. Nuclear extracts from T cells were prepared, and DNA-protein binding reaction was carried out for NF-AT as described under "Materials and Methods." The first set of four lanes shows the results of NF-AT analysis of extracts prepared from T cells which were incubated for 6 h in medium alone, PDBu/I, PDBu/I + CsA, or PDBu alone. A specific band representing NF-AT is seen only in the second lane; that is, in the extract of cells activated by the combination of PDBu and ionomycin. In the second set of lanes is the analysis of resting T cells activated for 30 min by addition of PDBu/I and of cells treated for 30 min with PDBu/I and subsequently incubated in medium alone for 2 h or 6 h, in medium with PDBu for 6 h, or in medium with PDBu and CsA (1 µg/ml) for 6 h. The samples were subjected to electrophoresis on 4% polyacrylamide gels containing 2.5% glycerol in 0.25 × TBE buffer. The gel was then dried, and bands were visualized by autoradiography. Confirmation that the shifted band was due to NF-AT (indicated by the arrow) was ascertained by competition assays as described under "Materials and Methods."

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REFERENCES