

IN SITU HYBRIDIZATION FOR INTERLEUKIN 2 AND INTERLEUKIN 2 RECEPTOR mRNA IN T CELLS ACTIVATED IN THE PRESENCE OR ABSENCE OF CYCLOSPORIN A

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An essential component in the response of T lymphocytes to mitogens and antigens is the expression of genes encoding lymphokines and IL-2-R. This induction can be monitored by hybridizing complementary DNA or RNA probes to the corresponding mRNA by the northern blotting technique (1). The latter requires large numbers of cells, and it does not provide direct information on the frequency of lymphocytes capable of expressing these genes or the level of mRNA per active cell. Such information is of concern in primary populations that consist of different lymphocyte subsets with distinct functional potentials. In situ hybridization potentially would allow one to use smaller numbers of cells and to detect induced genes at the level of individual lymphocytes.

In this paper, the mRNAs for IL-2 and the low-affinity IL-2-R have been detected by in situ hybridization of human T cells that have been stimulated with three different mitogens, PHA, anti-CD3, and anti-CD28, in conjunction with the tumour promoter PMA. For each of the mitogens, IL-2 mRNA is only found in a minority of the cells (20% or less) and for a short time, between 8 and 16 h after stimulation. In contrast, IL-2-R mRNA is present for a much longer time and in most cells. Using this approach I have analyzed the levels of IL-2 and IL-2-R mRNA in the CD4⁺ and CD8⁺ T cell subsets, and have examined the effects of adherent cells and cyclosporin A (CSA)¹ on lymphokine gene expression.

Materials and Methods

Materials. Leukocyte-rich buffy coats were purchased from the NY Blood Center; PHA-M was from Gibco Laboratories, Grand Island, NY; OKT3 anti-CD3 mAb was from Ortho Diagnostic Systems Inc., Westwood, MA; PMA from Sigma Chemical Co., St. Louis, MO; and Ficoll-Hypaque was from Pharmacia Fine Chemicals, Piscataway, NJ. Colleagues kindly supplied us with CSA (J. F. Borel and B. Ryffel, Sandoz Pharmaceutical, Basel, Switzerland) and the 9.3 anti-CD28 mAb (Dr. J. Laurence, Cornell University Medical Center, NY, and Dr. A. Moretta, Ludwig Institute for Cancer Research, Lausanne, Switzerland).

Cell Culture. Blood mononuclear cells were obtained by applying 10 ml of buffy coat preparations to 10 ml Ficoll-Hypaque. After centrifugation, the interface of mononuclear cells was washed and cultured at 10⁶ cells/ml in complete medium consisting of RPMI 1640 (Gibco Laboratories) supplemented with 5% heat-inactivated FCS (Sterile Systems, Logan, UT), 20 µg/ml gentamycin sulfate, 5 × 10⁻⁵ M 2-ME, and optimal levels of different mitogens: OKT3 at 30 ng/ml, PHA at 1%, PMA at 5 ng/ml, and 9.3 mAb at a 1:2,500 dilution of

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¹ Abbreviation used in this paper: CSA, cyclosporin A.

ascites fluid. To separate CD4 and CD8 subsets, nylon wool-nonadherent T cells were coated with OKT8 or OKT4 hybridoma culture supernatant, respectively (American Type Culture Collection, Rockville, MD), washed, and centrifuged onto Petri dishes (model 1005; Falcon Labware, Oxnard, PA) coated with anti-mouse Ig (Jackson Immunochemicals, Avondale, PA). The nonadherent cells were examined by FACS and found to be >99% depleted of the T cells subset that was reactive with the panning mAb. CD28⁺ cells, which lack the OKM1 or CD11b antigen (2), were enriched by panning nylon wool-nonadherent T cells coated with OKM1 hybridoma supernatant.

Cytocentrifuged Cell Preparations. Glass slides were coated for 3 h in Denhardt's medium (0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll Type 400, 3 × SSC) at 65°C, dipped briefly in H₂O, transferred to ethanol-acetic acid (3:1 vol/vol) for 20 min, and air dried. 10⁵ cells were cytopun for 3 min at 950 rpm onto the coated glass slides. The slides were air dried and immediately fixed in freshly prepared 4% paraformaldehyde in PBS without Ca²⁺ and Mg²⁺ (PD) for 20 min at room temperature. The slides were rinsed for 3 min in 3 × PD, and twice for 1 min in 1 × PD, and dehydrated for 5 min in graded ethanol solutions (50%, 70%, 95%, 100%). After air drying, the slides were stored at -20°C with dessicant.

Preparation of RNA Probes. The PST-1-PST-1 fragment from the c-DNA IL-2 clone pTCGF-5 (3) and the Sau-3A-Sau-3A fragment from the human IL-2-R clone (4) were subcloned into pIBI-30 vector (Int. Biotech. Inc., New Haven, CT). The recombinant plasmids were linearized with the appropriate restriction enzyme. Radioactive sense and antisense RNA probes were generated using T3 or T7 polymerase and ³⁵S-UTP (NEN; DuPont Co., Wilmington, DE). DNA templates were digested with DNase I (1 U/μg DNA; Promega Biotec, Madison, WI). The RNA probes were ethanol precipitated after adding 10 μg yeast tRNA. About 2 × 10⁶ cpm/μg DNA was obtained.

In Situ Hybridization. The fixed cells on slides were rehydrated, treated for 20 min at room temperature with 0.2 N HCl, washed in 1 × PD, and immersed in 2 × SSC for 15 min at 70°C. The slides were fixed for 5 min in freshly prepared 4% paraformaldehyde at room temperature, and washed in 3 × PD for 5 min and 1 × PD for 1 min. The slides were acetylated for 10 min in 0.1 M triethanolamine and 0.25% acetic anhydride, washed for 5 min in 2 × SSC, dehydrated, and allowed to dry. 20 μl of hybridization mixture was loaded on each slide. This contained 10 μl formamide, 2 μl 20 × SSC, 100 mM DTT, 2 μl of 10 mg/ml shared salmon sperm DNA, 2 μl of 10 mg/ml tRNA, 0.8 μl of nuclease-free BSA at 50 mg/ml, 0.2 μl of ribonucleotide-vanadyl complex (BioLabs, Beverly, MA) and 10⁶ cpm of ³⁵S-labeled probe. Siliconized coverslips were placed on top of the cells and sealed with rubber cement. The slides were incubated at 50°C overnight and then washed by incubating as follows: twice in 50% formamide in 2 × SSC for 30 min at 55°C, twice in 2 × SSC for 30 min at 55°C with gentle shaking, once at 37°C for 30 min in 2 × SSC containing 10 μg/ml RNase (Sigma Chemical Co.) and 10 U/ml RNase Ti (Boehringer Mannheim Biochemicals, Indianapolis, IN), twice in 50% formamide in 2 × SSC for 30 min at 55°C, and twice in 2 × SSC for 30 min at 55°C. Dehydrated slides were dipped in NTB2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with 0.6 M ammonium acetate. After 1-4 wk, the slides were developed in Kodak D19 for 5 min, rinsed for 1 min in tap water, and fixed for 5 min in Kodak fixer A. The slides were washed with tap water for 30 min and stained with Giemsa. Each data point represents the evaluation of four slides. Cells were considered positive for gene expression when there were at least five times as many grains as the cells with the highest background. Slides that were hybridized with labeled sense probes gave background labeling.

Results

Detection of IL-2 and IL-2-R mRNA by In Situ Hybridization. Blood mononuclear cells were stimulated with the combination of lectin, PHA, and the tumor promoter, PMA. The cells were examined by in situ hybridization using the optimal time points for IL-2 and IL-2-R mRNA expression as described previously with northern analysis (1). When an IL-2 probe synthesized in the antisense orientation (complementary to cellular mRNA) was used, a fraction of the cells hybridized strongly. When

the sense RNA probe was tested, only background labeling was noted (Fig. 1). Specific labeling with the antisense probe also was obtained when cells were probed for IL-2-R mRNA (Fig. 1).

Frequency of Cells Expressing IL-2 and IL-2-R mRNA: Kinetics and Comparison of Different Mitogens and T Cell Subsets. The kinetics of IL-2 mRNA induction, after stimulation of blood leukocytes with mitogens, was studied in CD4⁻ (CD8⁺, cytolytic) and CD8⁻ (CD4⁺, helper) T cell subsets. For either OKT3/PMA or PHA/PMA stimulation, cells expressing IL-2 mRNA could be detected within 4 h (Fig. 2, A and B). By 8–16 h of culture, there was a significant increase both in the number of labeled cells (Fig. 2) and the amount of IL-2 mRNA expressed by each cell, as judged by the number of grains counted per cell (not shown). The number of cells with IL-2 mRNA then decreased at later times. By 36 h of culture, no cells expressing IL-2 mRNA could be detected.

The expression of IL-2-R mRNA showed different kinetics from IL-2. No mRNA was detectable at 4 h. The signal then increased progressively with time reaching a maximum at 36 h where 30–50% of the cells were labeled (Fig. 2, C and D).

Both T cell subsets synthesized IL-2 mRNA when stimulated with either of 3 mitogens; PHA, anti-CD3, or anti-CD28 (Fig. 3). However, the CD8⁺ subset contained a lower frequency of cells with IL-2 mRNA, and the number of grains per cell was lower (Figs. 3 and 4).

Expression of IL-2 Transcripts in OKT3-stimulated T Cells: Effects of PMA and Adherent Cells. I next compared T cell proliferation, IL-2 production, and the frequency of CD4⁺ or CD8⁺ cells with IL-2 mRNA after stimulation with anti-CD3 mAb and different accessory signals. T cells stimulated with anti-CD3 and adherent cells proliferated to a similar extent to T cells stimulated with anti-CD3 plus PMA, or anti-CD3 plus PMA and adherent cells. However, the accumulation of IL-2 in the culture medium increased markedly with PMA (Table I). By in situ hybridization, a small but significant number of CD4⁺ and CD8⁺ cells were identified as positive

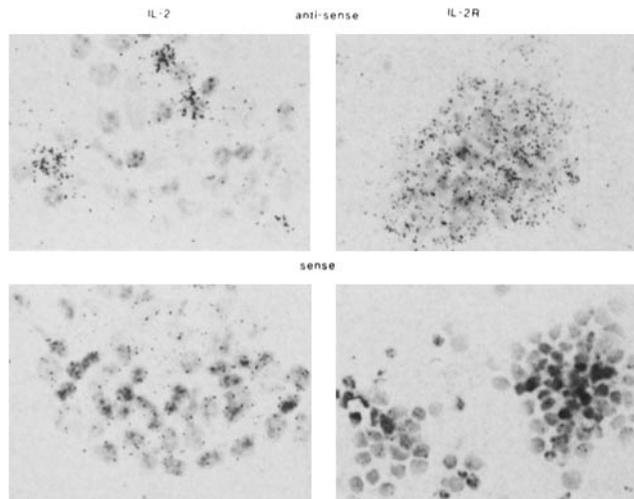


FIGURE 1. Detection by in situ hybridization of IL-2 and IL-2-R mRNA in mitogen-stimulated blood mononuclear cells. The cells were stimulated with PHA/PMA, and then hybridized with an antisense probe for IL-2 (*upper left*) or IL-2-R mRNA (*upper right*) or with a sense probe for IL-2 (*bottom left*) or IL-2-R mRNA (*bottom right*). Stimulation was 10 h for IL-2 and 24 h for IL-2-R. Shown here are three cells that were labeled heavily with the antisense IL-2 probe, and many more were labeled with the IL-2-R probe.

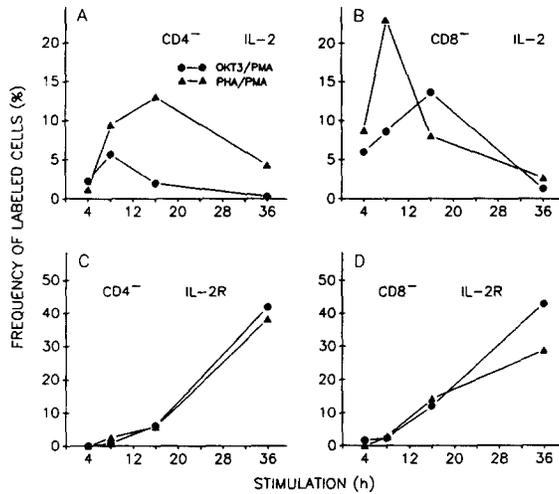


FIGURE 2. Kinetics of appearance of IL-2 and IL-2-R mRNA-bearing cells after stimulation with mitogens. Nylon wool-nonadherent CD4⁺ (CD8⁻) or CD8⁺ (CD4⁻) T cells were purified by panning with anti-CD8 or anti-CD4 mAb, and then the lymphocytes were stimulated with OKT3 (30 ng/ml) and PMA (5 ng/ml), or PHA (1%) and PMA. At the indicated times, the cells were analyzed for IL-2-R and IL-2 mRNA by in situ hybridization.

for IL-2 transcripts after stimulation with OKT3 mAb in the absence of PMA. However, PMA increased the frequency of cells expressing IL-2 mRNA dramatically as well as the number of copies of IL-2 mRNA cell (Table I). Therefore, it seems that IL-2 mRNA and IL-2 protein (activity) can be superinduced beyond that needed for lymphocyte DNA synthesis in culture.

CSA Blocks IL-2 but not IL-2-R Transcripts. The effect of CSA on the induction of IL-2 and IL-2-R mRNA by a number of stimuli was also analyzed by in situ hybridization. Confirming previous results obtained by northern blot (1, 5-7), 0.3-1 μg/ml CSA decreased the level of IL-2 transcripts when T cells were stimulated with PHA/PMA (Fig. 5) or anti-CD3 plus PMA (not shown). However higher concentrations of CSA (3 μg/ml) were necessary to obtain a 50-70% inhibition when the stimulus was anti-CD28 plus PMA (Fig. 6). Without CSA, 19% of the cells expressed IL-2 mRNA vs. 4-8% in its presence. It is noteworthy that the number of grains on the positive cells was reduced by CSA. The findings were confirmed by measuring IL-2 production. Synthesis of IL-2 was totally blocked on T cells stimulated with OKT3/PMA or PHA/PMA. However, the CD28 pathway was more resistant

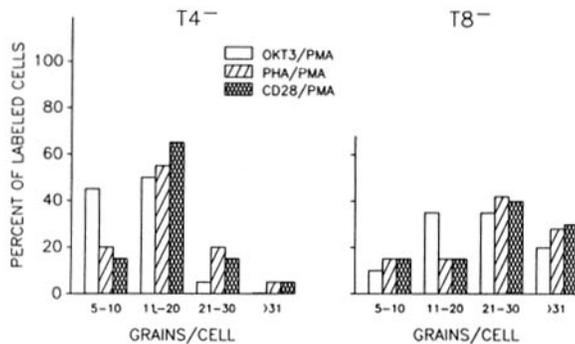


FIGURE 3. IL-2 mRNA accumulation in CD4⁺ and CD8⁺ cells stimulated with different mitogens. The cells were induced for 12 h with the indicated mitogens, and the number of grains were counted on positive cells.

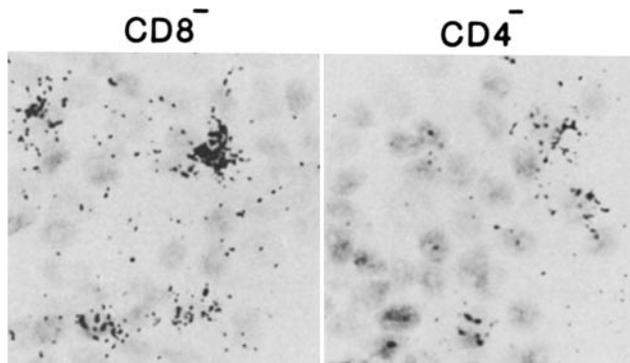


FIGURE 4. A comparison of IL-2 mRNA levels in CD4⁺ (CD8⁻) and CD8⁺ (CD4⁻) T cells. The lymphocyte subsets were prepared by panning with anti-CD8 and anti-CD4 mAb and then were stimulated for 12 h with PHA and PMA before analysis by in situ hybridization.

to CSA; 70% inhibition was obtained by exposing the cells to CSA for 30–60 min before stimulation (Table II).

In contrast to the effects on IL-2 mRNA, CSA did not reduce the level of IL-2-R mRNA (Fig. 5). Equal numbers of cells expressed IL-2-R mRNA in the presence and absence of CSA, and the distribution of grains at the single cell level was similar.

Discussion

The technique of in situ hybridization can be applied to visualize IL-2 and IL-2-R gene expression in activated T cells, even though these genes are expressed at relatively low levels. The approach offers an advantage over northern blot hybridization since one can obtain, on relatively small samples, information on the frequency of cells expressing a specific mRNA, as well as the amount of mRNA/cell. Northern blotting has been previously demonstrated that IL-2 is an inducible gene with a short mRNA half life (1). Less clear are the proportion of stimulated cells capable of expressing IL-2, the amount of IL-2 produced per cell, and the ability of different subpopulations of T cells to produce IL-2. The expression of the low-affinity IL-2-R at the single cell level can be analyzed with mAb such as anti-TAC (8), but to date only one study has reported visualization of IL-2 at the single cell level using antibodies (9).

The results with in situ hybridization show that a discrete number of activated T cells express IL-2 mRNA. Since the cells are not synchronized, and since IL-2 mRNA is rapidly turned on and off, the analysis represents the relative frequency of cells that at a given time express IL-2 transcript. IL-2 mRNA can be detected after 4 h of stimulation, and it peaks for just a short time between 8 and 16 h. Both subsets of T cells express IL-2 mRNA after stimulation with either PHA/PMA, α -CD3/PMA, or α -CD28/PMA. Yet the helper subset (CD8⁻) contained 2–3-fold more positive cells and more RNA (higher grain counts) per cell.

The data comparing DNA synthesis with the levels of IL-2 activity and IL-2 mRNA (Table I) raise interesting questions with respect to the amount of IL-2 one requires for optimal lymphocyte growth. It seems that under more physiologic conditions, such as the MLR (unpublished observations) and the use of accessory cells without PMA (Table I), one observes very low numbers of cells (0.5–3%) with IL-2 mRNA

TABLE I
Effects of Different Stimuli on IL-2 mRNA Expression and Growth of Human T Lymphocyte Subsets

Stimuli	Proliferation* [³ H]TdR uptake		IL-2 Production†		In situ hybridization‡				
	CD4 ⁺	CD8 ⁻	CD4 ⁺	CD8 ⁻	IL-2	CD4 ⁺	IL-2-R	IL-2	IL-2-R
None + adh	2	1.5	0	0	ND	ND	ND	ND	ND
OKT3 + adh	109	123	2.7	4.0	1.2 (15 ± 7)	19.1 (20 ± 10)	3.5 (25 ± 6)	18.8 (15 ± 8)	
PMA + adh	15	21	1.2	1.5	1.0 (15 ± 4)	19.0 (20 ± 10)	0.7 (15 ± 7)	20.0 (15 ± 7)	
OKT3/PMA + adh	115	157	51	64	18.8 (15 ± 6)	27.0 (33 ± 7)	13.1 (45 ± 20)	50.0 (25 ± 15)	
OKT3/PMA - adh	120	170	18	47	6.2 (15 ± 6)	33.3 (30 ± 10)	21.7 (25 ± 8)	42.2 (25 ± 15)	

¹⁰6 CD4⁺ or CD8⁻ T cells were cultured with or without 2 × 10⁵ plastic adherent cells (*adh*). The stimuli were OKT3 anti-CD3 mAb (30 ng/ml), PMA (5 ng/ml), or OKT3 and PMA.

* Thymidine was added during the final 4 h of a 72-h incubation period to 10⁵ cells.

† Supernatants collected after 16 h of initiation of culture were assayed for IL-2 activity using CCTL-2 cells for bioassay.

‡ After 10 h for IL-2 and 30 h for IL-2-R, cells were assessed for the expression of mRNA by in situ hybridization. Cells with more than five grains were scored as positive. The numbers represent the percentage of labeled cells, and in parentheses, the mean grain counts of positive cells.

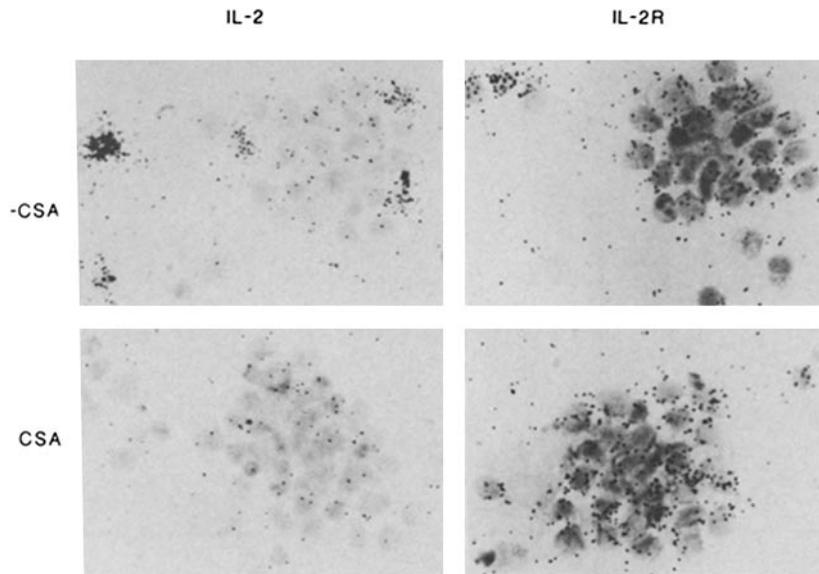


FIGURE 5. CSA reduces the levels of IL-2, but not IL-2-R, transcripts. Blood mononuclear cells were cultured with PHA/PMA in the presence (*bottom panels*) or absence (*top panels*) of CSA (1 $\mu\text{g}/\text{ml}$). At 8 h for IL-2, and 24 h for IL-2-R, the cells were analyzed for transcripts by in situ hybridization.

by in situ hybridization but high levels of DNA synthesis. Either a very low number of cells needs to make lymphokine for much larger numbers of cells with IL-2-R to respond, or many cells are making small amounts of IL-2 as an autocrine factor and the level of IL-2 mRNA is too low to detect by the in situ method. The use of accessory stimuli like PMA may lead to superinduction of IL-2 mRNA.

The expression of IL-2-R mRNA showed considerable differences compared with the expression of IL-2 mRNA. IL-2-R transcripts were present in 30–50% of cells after stimulation by only one stimulus like OKT3 or two stimuli, i.e., OKT3/PMA

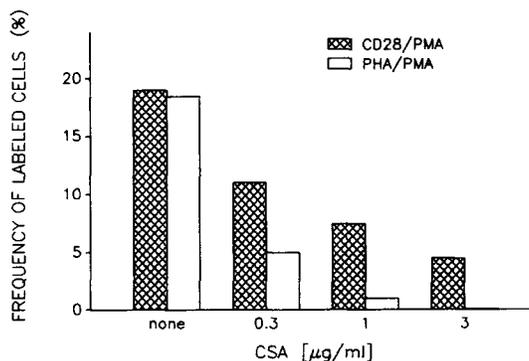


FIGURE 6. Effect of CSA IL-2 mRNA production on stimulated T cells. T cells were cultured for 8 h with 9.3 (anti-CD28) plus PMA or PHA plus PMA in the presence or absence of different concentrations of CSA. Cells were analyzed for IL-2 mRNA by in situ hybridization.

TABLE II
IL-2 Production by Stimulated T Cells: Effect of CSA

Stimuli	IL-2 production with CSA ($\mu\text{g/ml}$)			
	None	0.3	1	3
	<i>U/ml</i>			
CD28/PMA	53	35	18	10
OKT3/PMA	50	3	<1	<1
PHA/PMA	72	4	1	<1

T cells prepared as described in Materials and Methods were preincubated for 30 min with the indicated doses of CSA. Cells ($10^6/\text{ml}$) were stimulated with anti-CD28 (1:2,500 dilution of ascitic fluid), PHA (1%), or anti-CD3 (30 ng/ml) plus PMA (5 ng/ml). After 18 h, supernatants were collected and assayed for IL-2 production using CTLL-2 cells.

or PHA/PMA. Kinetic analysis showed that maximum expression of IL-2-R was achieved after 20–40 h of stimulation in contrast to IL-2, where the peak of expression was between 8 and 16 h. This suggests that the expression of IL-2 precedes expression of IL-2-R. At the present time we do not have information on the stability of these two mRNAs. Yet the results suggest that small numbers of cells making IL-2 could influence much larger numbers of cells with IL-2-R.

CSA blocks IL-2 at the level of nuclear transcription (1, 5), so it was not surprising to obtain similar results when cells stimulated with OKT3/PMA or PHA/PMA were analyzed by in situ hybridization. With CSA, the percentage of cells expressing IL-2 was dramatically reduced (from 20% to 0.1–1%), and the level of mRNA in the few positive cells decreased significantly as judged by grain counts. However, after stimulation with anti-CD28 and PMA, both IL-2 production and IL-2 transcripts were partially inhibited. This inhibition was achieved by incubating the cells for 1 h before stimulation with 3 $\mu\text{g/ml}$ CSA. Taken together, the data demonstrate that the CD28 pathways, although more resistant to the drug, are not completely resistant to CSA. These experiments were stimulated by the finding of June et al. (10), who recently reported that expression of IL-2 was CSA insensitive using anti-CD28 and PMA, but CSA sensitive using CD3 and CD28. In their work, CSA was used at 0.5–1.5 $\mu\text{g/ml}$ without a preincubation period. A physiologic ligand for CD28 has yet to be identified, so it is unclear whether the relative resistance of the CD28 pathway to CSA would be manifest as resistance to the drug in patients who are being immunosuppressed. CD28 antigen has been recently cloned (11), thus facilitating the study of its function.

In situ hybridization confirmed prior observations (1) on the insensitivity of IL-2-R mRNA expression to CSA. The frequency of cells with IL-2-R transcript was not changed by this drug, and the number of grains counted on positive cells was not altered as well.

In situ hybridization could become an important tool for monitoring lymphocyte activation in vitro and in vivo. A concern about studies of lymphokine gene expression in vivo is that the technique may not be sensitive enough to detect biologically relevant levels of IL-2, either because the level of mRNA per cell is too low, or because very few IL-2-producing cells are required to mediate T cell responses (Table I).

Summary

RNA-RNA in situ hybridization was used to study the frequency of cells producing mRNA for IL-2 and IL-2-R in T lymphocytes stimulated by either of three mitogens: anti-CD3, anti-CD28, or PHA. Both CD4⁺ and CD8⁺ T cells expressed transcripts for IL-2 and the low-affinity IL-2-R when stimulated with these mitogens plus PMA. IL-2 transcripts peaked at 8–16 h, and IL-2-R at 24–40 h. Cyclosporin A (CSA) inhibited the synthesis of IL-2, but not IL-2-R mRNA, after stimulation by PHA or anti-CD3. However, higher concentrations of CSA were necessary to achieve 50–70% inhibition after stimulation with anti-CD28. At optimal points 12–22% of CD4⁺ and 5–13% of CD8⁺ cells expressed IL-2 mRNA, while 30–50% of cells of both subsets had IL-2-R mRNA. The IL-2 grain counts, which relate to the level of mRNA/cell, were higher in the CD4⁺ subset but could be increased several fold in the CD8⁺ subset in the presence of adherent accessory cells. The use of PMA as an accessory stimulus, in addition to adherent cells, greatly increased the frequency of lymphocytes with IL-2 mRNA and the amount of IL-2 activity in the culture medium, but the proliferative response was not significantly boosted. These observations indicate at the single cell level that many CD4⁺ or CD8⁺ lymphocytes can make IL-2 mRNA, and that the induction of IL-2 with several stimuli is reduced by CSA and enhanced by PMA.

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