LYMPHOKINE GENE EXPRESSION IN VIVO IS INHIBITED BY CYCLOSPORIN A

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Following mitogenic or antigenic stimulation in vitro, T lymphocytes are induced to express a large number of genes, including those encoding lymphokines and IL-2 receptors (1–4). The induction of lymphokine genes is particularly sensitive to the immunosuppressive drug cyclosporin A [CSA][1] (1, 5–8). Since responsiveness to IL-2 is induced in the presence of CSA, the loss of growth factors and other lymphokines is thought to be the main mechanism for the immunosuppressive effects of CSA in vivo (9). However, much of the work on CSA has involved in vitro approaches. Molecular approaches have not been used in vivo, and recent evidence suggests that CSA does not even block DNA synthesis in mice or rats (10, 11). I have looked into these unknowns using a protocol wherein the injection of allogeneic cells induces the formation of cytolytic T cells in vivo (10, 12, 13). This response involves IL-2, since it is blocked by a neutralizing anti-IL-2 antibody (13). I have been able to document the induction of lymphokine genes in vivo and to determine that CSA can inhibit proliferative responses in mice whether allogeneic cells or mitogens are the stimuli.

Materials and Methods

Materials. [CxD2]F_1 and B6xD2 mice were obtained from Trudeau Institute (Saranac Lake, NY) and Con A from Miles Laboratories Inc. (Elkhart, IN). Ascites containing anti-CD3 mAb was obtained by injecting 2×10^7 2C11 anti-CD3 hybridoma cells (14) intraperitoneally into pristane-primed CxD2 F_1 mice. Drs. J. F. Borel and B. Ryffel (Sandoz Pharmaceutical, Basel, Switzerland) and Sandoz (East Hanover, NJ) generously supplied CSA.

CSA Treatment. Mice were treated orally with CSA (30 mg/kg/day). Mice were immunosuppressed with CSA 48 h before treatment with alloantigens or mitogens. CSA levels in serum were determined to be 900–3,500 ng/ml using an RIA kit (Sandoz, Basel, Switzerland).

Stimulation of Popliteal Lymph Nodes and Cell Preparations. Popliteal lymph nodes were injected as described (13). 30 μl of PBS containing 10^7 B6xD2 spleen cells was injected into the left hind foot pad of [CxD2]F_1 recipients. As a control, the right hind foot pads were injected with 30 μl of PBS or 30 μl containing 10^7 syngeneic cells. Alternatively, hind foot pads were injected with 50 μl of Con A (1 mg/ml) or 30 μl of 2C11 ascites fluid. At the appropriate time, the draining popliteal lymph nodes were removed, and cell suspensions were prepared. 10^6 cells/ml were left unstimulated or were stimulated with graded doses of rIL-2 (1.2×10^6 U/mg; Biogen, Cambridge, MA). Proliferation and response to IL-2 were assessed

This work was supported by grant GM-37643 from the National Institutes of Health. Address correspondence to Dr. A. Granelli-Piperno, The Rockefeller University, 1230 York Avenue, Box 280, New York, NY 10021.

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

on triplicate cultures of 10^5 cells that were pulsed for 4 h with 1.5 μCi [^{3}H]TdR either at 0–4 or 24–28 h of culture.

Northern Blot Analysis. The draining lymph nodes (8–10 nodes for each time point) were carefully dissected, cleaned of connective tissues, and immediately immersed in 4 M guanidine isothiocyanate. Nodes were disrupted by sonication using a Polytron, and total RNA was isolated by centrifugation through a cesium chloride cushion (15, 16). 10 μg of RNA was electrophoresed on a 1% or 1.5% agarose-formaldehyde gel. RNA was transferred by vacuum to gene screen filters (New England Nuclear, Boston, MA) and crosslinked by UV (1200 μJ) to the filters for hybridization with ^{32}P-labeled cRNA probes.

Preparation of cRNA Probes. The Pst I fragments from the cDNA IFN-γ clone pms10 (17), and pm IL-2R-1 (18) were subcloned into pIBI-30 vector (Institute of Biotechnology Inc., New Haven, CT). The RSA fragment of the cDNA pm IL-2 (19) was adapted with ECO RI linkers and subcloned into the ECO RI site of Bluescript KSII$^+$ (Stratagene, La Jolla, CA). The recombinant plasmids were linearized and radioactive sense and antisense probes were generated using T3 and T7 polymerase and [^{35}S]UTP or [^{32}P]CTP (NEN DuPont Co., Wilmington, DE).

In Situ Hybridization. Lymph node cell suspensions were cytocentrifuged on coated glass slides as described (20); alternatively, lymph nodes were embedded in OTC and frozen cryostat sections were applied to 0.5% gelatin slides. Cells and sections were fixed for 20 min in 4% paraformaldehyde at room temperature and processed with radiolabeled antisense and sense RNA probes as described (20).

Immunocytochemistry and Autoradiography. Lymph node cell suspensions were pulsed with [^{3}H]TdR for 2 h. Cells were washed three times, resuspended in PBS, and cytospun. Cells were fixed in 2% buffered glutaraldehyde and dehydrated. Alternatively, [^{3}H]TdR (25 μCi) was injected intraperitoneally into mice. After 2 h popliteal lymph nodes were harvested and tissues were embedded with OTC (Tissue Tek). Serial 6-μm sections were cut in a cryostat and picked up on multiwell slides (Carlson Scientific Inc., Peotone, IL), air-dried, and stored at −20°C. Sections were dried at 37°C, fixed with acetone (10 min at room temperature), and stained with mAb culture supernatants obtained via the American Type Culture Collection (B220 TIB-146 for B cells and L3T4 TIB-207 for T cells). After washing, sections were incubated with peroxidase-labeled mouse anti-rat IgG. The bound anti-Ig was visualized by the immunoperoxidase Vectastain ABC kit (Vector Laboratories). Dehydrated slides were dipped in NTB-2 emulsion [Eastman Kodak, Rochester, NY). After various times, the slides were developed in Kodak D19 for 5 min, rinsed in tap water, and fixed for 5 min in Kodak fixer A.

Results

Effects of CSA on the Proliferative Responses Induced by Foreign Cells In Situ. Popliteal lymph nodes were stimulated by the administration of allogeneic spleen cells (H-2^{bxd} cells into the footpads of H-2^d recipients), in the presence or absence of the immunosuppressive agent, CSA. The draining lymph nodes were removed at daily intervals, and the cell suspensions were assessed for DNA synthesis before and after in vitro stimulation with rIL-2. As seen in Fig. 1, *A* and *B* (●), an increase in DNA synthesis was observed in lymph node cells taken 48–72 h after exposure to allogeneic leukocytes. This proliferative response was much reduced after treatment with CSA (Fig. 1 *A*, compare ● with ▲).

The administration of allogeneic cells also increased the responsiveness of the draining lymph node cells to exogenous IL-2 (Fig. 1 *B* ○, △). However, the development of IL-2 responsiveness was not reduced by CSA. Only low doses of IL-2 were required to stimulate DNA synthesis (Fig. 1 *C*), suggesting the presence of functional high affinity IL-2 receptors.

We also isolated popliteal nodes from mice that had been exposed to mitogens, comparing these with injections of syngeneic or allogeneic cells. Mitogens, like al-

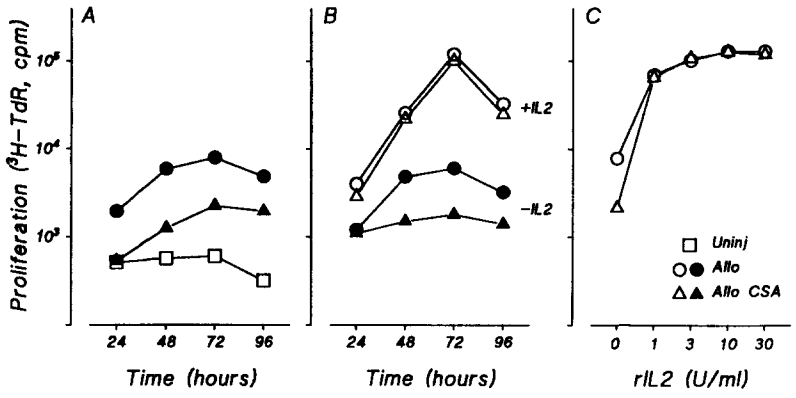

FIGURE 1. Effect of CSA on the proliferative responses of popliteal lymph node cells activated in vivo. 10^7 B6xD2 (H-2^{bxd}) spleen cells were injected into the hind footpads of CxD2 mice (H-2^{dxd}) that were treated (Δ, ▲) or not treated (○, ●) with CSA. The draining nodes were removed at the indicated time points and cell suspensions were prepared. (*A*) 10^5 cells were immediately pulsed for 4 h with [^{3}H]TdR to measure spontaneous proliferation. (*B*) 10^5 cells were cultured with/without IL-2 (10 U/ml) for 24 h and then pulsed with [^{3}H]TdR for 4 h. (*C*) After 72 h of activation by alloantigen in vivo, 10^5 lymph node cells were cultured with different concentrations of IL-2 for 24 h and then pulsed with [^{3}H]TdR for 4 h.

loantigens, increased the level of DNA synthesis in the draining lymph node, and this increase was suppressed 60–80% if the animals had been given CSA (Table I, left side). Each stimulus also resulted in a heightened T cell response to IL-2 in vitro; but the enhanced responsiveness was largely resistant to CSA (Table I, right side). These results indicated that CSA was having similar effects in vivo to those observed in vitro, i.e., IL-2 responsiveness was being induced but DNA synthesis was blocked (1, 21).

Identification of Proliferating Cells in Lymph Nodes Stimulated In Situ. To better identify and enumerate cells that were responding to allogeneic or mitogenic stimulation, we administered [^{3}H]TdR in vivo, or for 2 h in vitro immediately after isolation

TABLE I

Response of Lymph Node Cells to IL-2 After In Vivo Stimulation

Stimulus in vivo	Without IL-2		With IL-2 (10 U/ml)	
	No CSA	With CSA	No CSA	With CSA
	cpm		*cpm*	
None	800 ± 30	ND	2,430 ± 70	ND
Anti-CD3	4,616 ± 150	939 ± 7	32,322 ± 1,100	24,661 ± 800
Con A	7,248 ± 170	2,592 ± 80	38,216 ± 1,000	29,438 ± 750
Allogeneic cells	7,500 ± 130	2,346 ± 54	58,286 ± 1,300	51,587 ± 1,200
Syngeneic cells	1,583 ± 100	1,300 ± 90	16,768 ± 750	14,685 ± 1,000

Anti-CD3 mAb (30 μl of 2C11 ascites fluid), Con A (50 μg), B6xD2 (10^7) cells, and CxD2 F_1 (10^7) cells were injected in the hind foot pad of CxD2 F_1 mice. The mice were or were not treated with CSA. After 48 h, the draining lymph nodes were collected and cell suspensions were prepared. Cells were plated at 10^6 cells/ml in the presence or absence of rIL2 (10 U/ml). After 24 h of culture, proliferation was determined in 100-μl aliquots by adding [^{3}H]TdR for 4 h.

of the draining lymph node cells. The specimens were processed for autoradiography. 2–3% of the cells were labeled in the suspensions from unstimulated mice, but much larger percentages were synthesizing DNA after stimulation with allogeneic cells or mitogens, especially at later time points (Table II). When the cells were treated with either anti-B220 (B cell-specific) or anti Thy-1 (T cell-specific) antibodies and complement just before measuring [^{3}H]TdR uptake, the proliferating cells were found to be T cells (Fig. 2).

When [^{3}H]TdR was given in vivo to mice, an increase in [^{3}H]TdR labeled profiles was apparent in autoradiograms of nodes that had been stimulated with allogeneic cells. When the lymph node sections were stained with anti-B or anti-T cell mAbs and processed for autoradiography, the labeled cells were for the most part in the T areas (para and deep cortex with many CD4$^+$ B220$^-$ cells) of the lymph node (Fig. 3).

The effect of CSA on all of the above parameters was monitored. CSA diminished but did not completely block [^{3}H]TdR uptake in lymph node cells that had been stimulated in vivo with allogeneic cells or mitogens (Table II). CSA also reduced,

TABLE II

[^{3}H]TdR-labeled Lymph Node Cells after In Vivo Stimulation

	Percent of labeled cells	
Stimulus in vivo	24 h	48 h
Uninjected	2.5	2.0
Allo	7.6	25.0
Allo CSA	2.9	7.8
Anti CD3	ND	40.0
Anti CD3 CSA	ND	15.0
Con A	35.0	41.8
Con A CSA	3.2	8.8

10^7 allogeneic cells (B6xD2 mice), anti-CD3 mAb (30 μl 2C11 ascites), or Con A (50 μg) were injected in the hind foot pads of CxD2 F_1 mice. At the indicated times, the draining lymph nodes were collected and cell suspensions were prepared. Cells were pulsed for 2 h with 0.5 μCi of [^{3}H]TdR and processed as described in Materials and Methods.

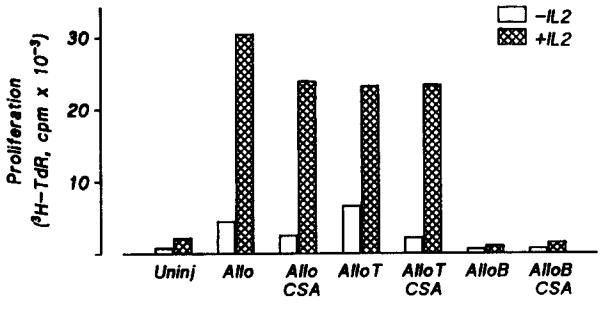

FIGURE 2. Characterization of the proliferating lymphocytes after alloantigen stimulation in vivo. Allogeneic spleen cells were injected into the hind foot pads of mice that were treated or not treated with CSA. After 72 h the draining lymph nodes were removed and cells suspensions were prepared. Cells were left untreated or depleted of B cells by treatment with anti-B220, or depleted of T cells by treatment with Thy-1 and complement. 10^5 cells were cultured with IL-2 (10 U/ml) for 24 h and then pulsed with [^{3}H]TdR for 4 h.

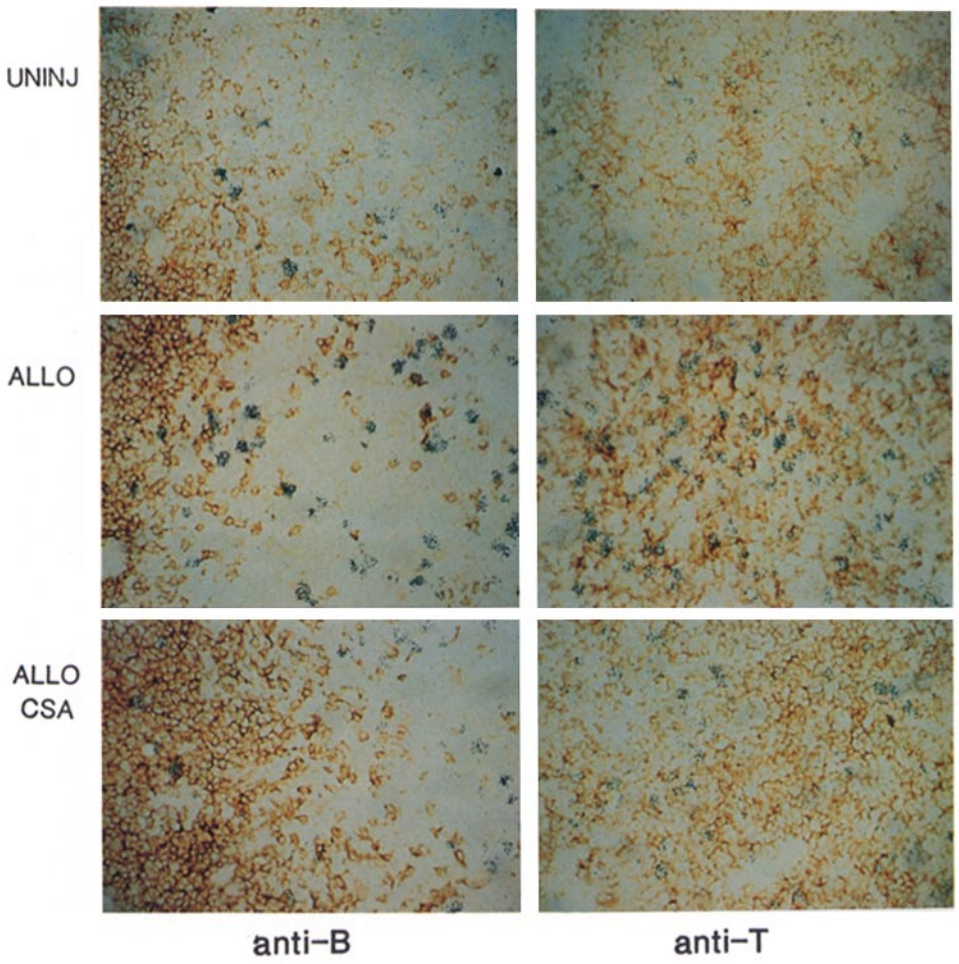

FIGURE 3. Localization of cells synthesizing DNA in situ. Mice were stimulated with alloantigen and at 48 h, [^{3}H]TdR was injected. 2 h later lymph nodes were taken and sections were prepared for immunolabeling with mAb to B or T cells followed by peroxidase mouse anti-rat Ig and diaminobenzidine staining. Shown here is the B220 mAb to B cells (*left panels*) and αCD4 to T cells (*right panels*). After dehydration, sections were processed for autoradiography. Note that the [^{3}H]TdR-labeled cells are predominantly confined to the T area, which in these sections are the areas of cortex with few B220$^+$ cells and many CD4$^+$ cells. (*Top*) Representative sections from control mice; (*middle*) sections from mice stimulated with alloantigen; and (*bottom*) sections from mice stimulated with allo in the presence of CSA.

but did not eliminate the number of labeled cells in lymph node sections (Fig. 3). For example, an average of 85 labeled cells were scored on lymph node sections from uninjected mice, 390–410 on lymph node sections from alloantigen-injected mice and 140–160 on sections from CSA-treated mice. At this time we do not know if the cells that incorporated [^{3}H]TdR in the presence of CSA progressed normally through the cell cycle or were arrested in S phase because of a lack of lymphokine.

Expression of T Cell Activation Genes after In Vivo Stimulation. RNA was extracted

from lymph nodes at different time points after allogeneic stimulation and analyzed by Northern blot. A strong signal for IFN-g mRNAs was noted upon administration of alloantigen, with maximal expression at 22–36 h (Fig. 4). By 48 h, transcripts were no longer detected (Fig. 4). The induction of transcripts was dependent upon the dose of alloantigen from 1 to 10 × 10^6 allogeneic spleen cells (Fig. 5). Stimulation with syngeneic cells, even at high concentrations, did not induce IFN-g (see below).

Injection of allogeneic cells induced IL-2 mRNA as well (Figs. 4 and 5). With the IL-2 probe used, two bands were detected: a fast migrating band at 1 kb corresponding to the transcript for IL-2 that is present only in stimulated cells both for

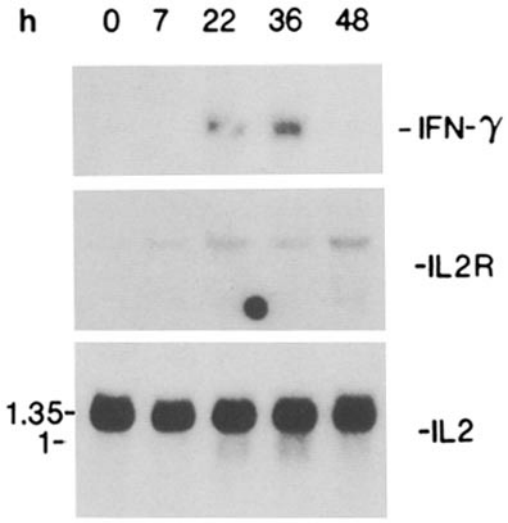

FIGURE 4. Time course of mRNA accumulation in the draining lymph node. Allogeneic spleen cells were injected into the hind footpads of mice. The draining nodes were removed at the indicated times and RNA was extracted. 10 μg of RNA was separated on 1% agarose-formaldehyde gel and hybridized with ^{32}P-labeled probes.

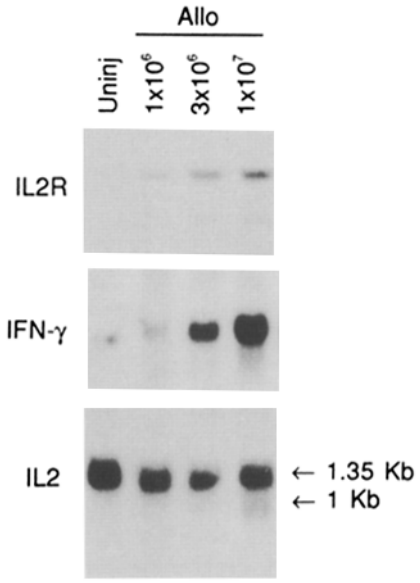

FIGURE 5. Lymphokine mRNA expression. Effect of the number allogeneic cells injected. The hind footpads of mice, either immunosuppressed or not with CSA, were injected with graded doses of allogeneic spleen cells. After 24 h, lymph nodes were collected and total RNA was separated on 1% agarose-formaldehyde gel and hybridized with ^{32}P-labeled probe.

lymph nodes and spleen, and a slow migrating band (1.35 kb) that is present both in unstimulated and stimulated cells (Figs. 4 and 5). Removal of the DNA template after transcription of the RNA probe eliminated the 1.35-kb band. Routinely, the cRNA probe was used without removing the DNA template because the 1.35-kb band served as an internal control, demonstrating that the same amount of RNA was loaded in each lane. As was seen for IFN-γ, IL-2 expression was observed at 22–36 h (Fig. 4). P55 IL-2R mRNA was also induced upon injection of allogeneic cells (Fig. 4).

The experiments were also conducted using mitogens as the stimuli in vivo. Both Con A and anti-CD3 mAb induced the mRNAs for p55 IL-2 receptor, IFN-γ, and IL-2 (Fig. 6). With Con A stimulation, lymphokine mRNA appeared as early as 5 h, and peaked at 24 h (data not shown).

We next monitored the effects of CSA on lymphokine gene expression in vivo. CSA strongly blocked the induction of IFN-γ and IL-2 mRNA but only partially blocked the induction of the p55 IL-2 receptor message (Figs. 6 and 7). Taken to-

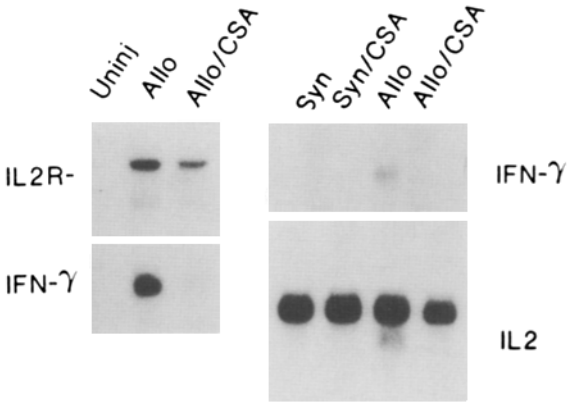

FIGURE 6. Effect of CSA on lymphokine mRNA expression. Two experiments in which syngeneic (CxD2F1) or allogeneic (B6xD2F1) spleen cells (10^7) were injected into the hind footpads of CxD2 mice that were or were not immunosuppressed with CSA. After 24 h for IL-2 and IFN, and 48 h for IL-2R, lymph nodes were collected. Total RNA was extracted and separated on 1% agarose-formaldehyde gel and hybridized with ^{32}P-labeled probes.

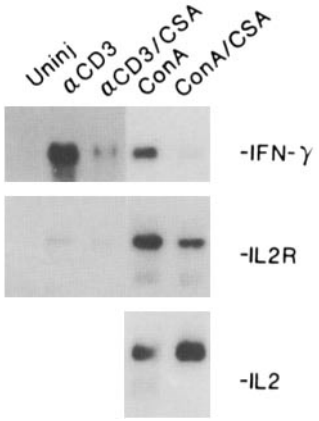

FIGURE 7. Effect of CSA on lymphokine mRNA expression. Anti-CD3 mAb (2C11) ascites fluid (30 μl) or Con A (50 μg) was injected into the hind footpads of CSA or non-CSA treated mice. After 24 h for IL-2 and IFN, and 48 h for IL-2R, lymph nodes were collected. Total RNA was extracted and separated on 1% agarose-formaldehyde gel and hybridized with ^{32}P-labeled probes.

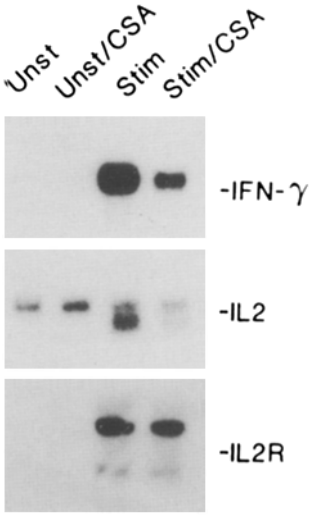

FIGURE 8. Treatment with CSA in vivo inhibits lymphokine mRNA on in vitro-stimulated spleen cells. Spleen cells obtained from mice that were or were not immunosuppressed with CSA were left unstimulated or stimulated in vitro with Con A (5 μg/ml) and PMA (5 ng/ml). After 5 h RNA was extracted separated on agarose-formaldehyde gel and hybridized with ^{32}P-labeled probes.

TABLE III

In Situ Hybridization for IL-2, IL-2R, and IFN-γ mRNAs

	IL-2		IFN-γ		IL-2R	
Time	Allo	Allo-CSA	Allo	Allo-CSA	Allo	Allo-CSA
h	%		%		%	
7	0	ND	ND	ND	ND	ND
16	0.8	0.2	ND	ND	ND	ND
24	2.5	0.7	3.5	0.11	0.9	0.8
36	2.0	0.4	2.1	0.16	4.6	3.3
48	0	0	0.7	0	5.9	4.0

Allogeneic spleen cells (10^7) were injected in the hind foot pads of mice treated or not with CSA (30 mg/kg/day). At the indicated times, the draining lymph nodes were removed and cell suspensions were prepared. Cells were cytospun and analyzed for mRNAs by in situ hybridization. The numbers represent the percentage of labeled cells. Cells with more than 10 grains were scored as positive. Background was 2.4 grains per cell.

gether, these results show that in vivo stimulation with allogeneic cells or mitogens induces several T cell activation genes, and this induction is greatly reduced by CSA.

To evaluate the reversibility of CSA, we administered the drug in vivo on three consecutive days and then removed spleen cells to test their response to a subsequent challenge of mitogen in vitro. Immediately upon isolation, the cells were less responsive to the inducing effects of mitogen (Fig. 8), i.e., the induction of IFN-γ or IL-2 mRNA was decreased ~20-fold. However this inhibition was short lived. If the cells were cultured for 20 h before challenge, the cells from CSA-treated animals showed no block in the induction of IL-2 mRNA. Therefore, CSA seems to partially block the response of T cells from CSA-treated mice to mitogens in vitro, but this block is rapidly reversible.

Frequency of Cells Expressing Activation Genes In Vivo. In situ hybridization was used

to identify cells expressing T cell activation genes at the single cell level. After injection of allogeneic cells, we monitored transcripts both on dispersed lymph node cells or in lymph node sections. IL-2 and IFN-γ transcripts peaked at 24–36 h, while IL-2 receptor transcripts appeared later and were more evident at 48 h (Table III). 2–4% of the cells were labeled at the peak of the response with lymphokine probes, and 4–6% with the IL-2 receptor probe (Table III). Administration of CSA reduced the number of cells that were induced to express lymphokine, but not IL-2 receptor, genes (Table III).

Discussion

Studies in tissue culture have identified several molecular events that are altered in the immunologically stimulated T lymphocyte. No less than 70 genes are reported to be induced (3, 4). We have used single cell and molecular approaches to monitor some of the main parameters of T cell activation in situ, i.e., upon challenge of mice with foreign cells or with mitogens. Our results show that several mRNAs that underlie T cell function can be induced in vivo. These are the mRNAs for the lymphokines IL-2 and IFN-γ, and for the low affinity IL-2 receptor. High affinity IL-2 receptors are also present in mice challenged with foreign cells, since there is a large increase in the responsiveness of the draining lymph node to very low doses of exogenous IL-2 (Fig. 1). The number of cells with lymphokine transcripts does not exceed 2–3% in the response to alloantigen, while somewhat higher percentages, ~5%, express the p55 IL-2 receptor mRNA at the assay times we tested. Prior work has shown that a polyclonal neutralizing antibody to IL-2 can block T cell responses in situ and in vitro (13). Taken together then, many of the molecular events that are occurring in tissue culture also occur in vivo upon challenge with foreign leukocytes and mitogens.

The area in which there *seems* to be a discrepancy between events in tissue culture and experimental animals pertains to the mechanism of action of the immunosuppressive drug CSA. There are reports that indicate that CSA does not block certain parameters of T cell activation in situ (10, 11, 22) and that DNA synthesis still proceeds normally in response to alloantigen (10). Yet in vitro, the block in DNA synthesis that is induced by CSA is profound (1, 21, 23–26). The latter primarily involves a block in the induction of lymphokine mRNAs and not a block in the acquisition of IL-2 responsiveness (1, 21, 27). To a large extent, my findings indicate that the action of CSA in vivo is similar to its action in vitro. There is a block in the induction of both IL-2 and IFN-γ mRNAs, and there are reduced numbers of cells synthesizing DNA. These changes can be visualized in tissue sections of the stimulated lymph node and in freshly prepared cell suspensions. When mitogens instead of alloantigens are used as stimuli in vivo, I likewise observe that CSA reduces lymphokine gene expression and DNA synthesis (Table II) and again CSA does not block the induction of IL-2 responsiveness (Fig. 1).

The principal unknown therefore is to explain the fact that the reduction in DNA synthesis in vivo is less complete than in vitro. This could reflect the existence of a CSA-resistant IL-2 induction pathway. One such pathway entails the stimulation of T cells with anti-CD28 mAb (20, 28). The physiologic ligand for CD28 is not yet known, and a recent report suggests that anti-CD28 increases lymphokine mRNAs at the level of mRNA stability rather than induction of transcription (29). Another

possibility is that there are CSA-resistant, IL-2-independent mechanisms for T cell growth. IL-2 independence is sometimes difficult to establish. For example, it seemed that T cells stimulated with anti-CD3 mAb and monocytes did not produce IL-2 (30), yet a role for IL-2 was apparent when poly(A) mRNA rather than total RNA was used to detect the IL-2 transcripts, or when anti-IL-2 receptor antibody was used to block DNA synthesis (31). Other growth factors like IL-4 are known, but to date these are sensitive to CSA (32). A third possibility is that in mice the clearance or catabolism of CSA is rapid, and/or the drug does not gain as ready access to T cells. Given the fact that the effects of CSA are rapidly reversible, it may be difficult to achieve adequate levels of the drug continuously in lymphoid organs. It is of interest that the dose of CSA that must be given to mice is high relative to that used clinically. In our experiments, we had to achieve serum levels of close to 1 μg/ml to detect immunosuppression at the level of the draining lymph node, i.e., in the induction of alloreactive CTL. In patients, serum levels of 100 ng/ml are sufficient to maintain immunosuppression.

Therefore, the data in this article indicate that CSA can influence lymphokine gene expression in mice as it does in tissue culture systems, and does so rather selectively when compared with the lack of an effect on the acquisition of IL-2 responsiveness. However, it is not yet clear if the block in lymphokine gene expression is sufficient to account for suppression of immune responses in vivo (23, 24, 33–35) or if there are additional pathways that remain to be identified.

Summary

Murine T cells were stimulated in vivo by administering allogeneic cells or mitogens into the foot pads and then examining the draining popliteal lymph nodes. Allogeneic spleen cells induced the expression of IL2 and IFN-γ mRNAs in a time- and dose-dependent manner. Induction of these transcripts also was detected after administration of Con A and anti-CD3 mAb. An increase in DNA-synthesizing cells was observed by 48 h, and these were shown to be T cells because of their sensitivity to anti-Thy-1 but not anti-B220 mAb and complement, and because of their localization to the T-dependent areas of the lymph node.

The in vivo administration of cyclosporin A (CSA) reduced several of these T cell responses. The level of DNA synthesis and the frequency of cells synthesizing DNA were decreased by ~75%, while the induction of IL-2 responsiveness was not substantially diminished. IL-2 and IFN-γ transcripts were inhibited at least 70–90%, as determined by Northern blot and in situ hybridization. Although the inhibition by CSA was not as complete in animals as observed previously in tissue culture, our findings indicate that in both systems, a major site of action of CSA is to inhibit T cell growth by inhibiting lymphokine production.

I thank Dr. M. Suthanthiran for performing the RIA for measuring the level of CSA in mouse serum, and Dr. R. M. Steinman for critical review of the manuscript and helpful discussions. I also thank Ms. A. Skomorowski and S. Lane for expert technical assistance.

Received for publication 23 October 1989.

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