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Pharmacology of Cyclosporine

VI. Cellular Activation: Regulation of Intracellular Events by Cyclosporine

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† Abbreviations used are: CS, cyclosporine; TR, T cell receptor; PK-C, protein kinase C; (m)RNA, (messenger) ribonucleic acid; RNase, ribonuclease; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; IL, interleukin; CSF, colony-stimulating factor; NF, nuclear factor; PE, phorbol ester; ARRE, antigen receptor response elements; PPIase, peptidyl-prolyl-*cis-trans*-isomerase.

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A. Introduction

THE interaction of antigen in the proper histocompatibility context with the T lymphocyte antigen receptor leads to biochemical events resulting in proliferation, differentiation and maturation of T lymphocytes with specific immunological functions, e.g., helper and cytotoxic-suppressor functions. A schematic representation of the cellular interactions and cytokines produced upon activation of the immune system is shown in fig. 1. The T lymphocytes play a central regulatory role in this biological response. Other levels of controls occur at the level of antigen-presenting cells (antigen processing and presentation), B lymphocytes (differentiation and maturation), and the lymphoid precursor cells. The main target of cyclosporine (CS)† action is the T lymphocyte, although other cell lineages are not completely insensitive to the drug. The unique features of CS that have been detected by Borel et al. (11–13) and which are still valid can be summarized as follows: (*a*) potent in vitro and in vivo immunosuppressive activity, (*b*) immunosuppressive activity in all tested species, (*c*) reversibility of pharmacologic effect (no cytotoxic activity), (*d*) inhibition of cell activation at an early phase of the cycle, and (*e*) specificity for lymphocytes, no inhibition of hemopoiesis.

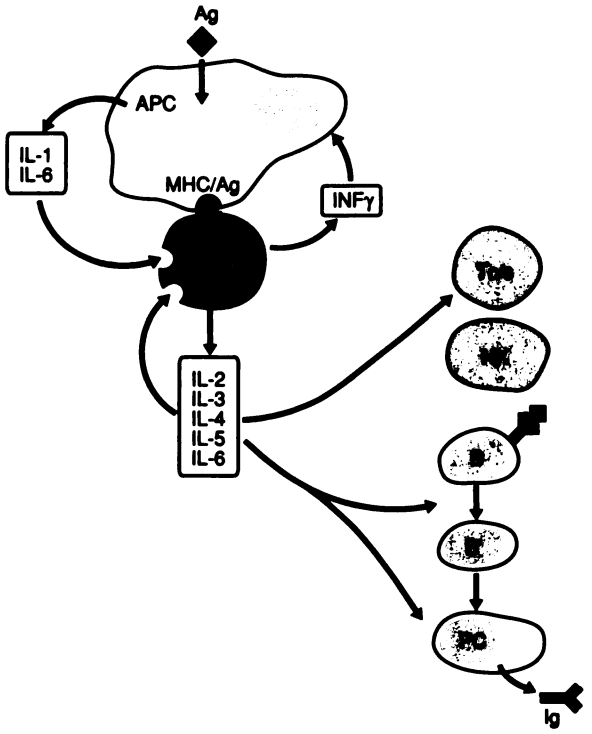

FIG. 1. Cellular interactions for lymphocyte activation, proliferation, and differentiation. Antigen (Ag) processed by antigen-presenting cells (APC) is recognized in the context of major histocompatibility (MHC) class II structures by the antigen receptors (TR) on T-helper lymphocytes (T_H). Subsequently, cytokines [interleukin, IL-1 to IL-6, interferon-γ (INFγ)] are synthesized by APC and T_H. These cytokines have multiple regulatory effects, e.g., autocrine stimulation of T_H and differentiation of cytotoxic and suppressor T lymphocytes (Tc/s), natural killer cells (NK), and B lymphocytes. PC, plasma cells; Ig, immunoglobulin.

In this chapter, biochemical and genetic events of T cell activation and its modulation by CS are summarized. Although the main focus is on intracellular aspects, a few overlaps with the review by Di Padova were not avoidable (this issue).

B. T Lymphocyte Activation

Activation of T lymphocytes by antigen and a variety of mitogenic ligands results in proliferation and differentiation into effector cells. The multiple steps involved are signal recognition, signal transduction, gene activation, expression of growth factor receptors, and growth factor synthesis.

1. Signal Recognition

T lymphocytes. Binding of antigen at the T cell receptor (TR) is an absolute requirement for T cell activation under physiologic conditions. Although antigen binding has not been directly studied, present experimental evidence with ligands binding to TR does not suggest any effect of CS at the level of ligand recognition: the binding of an anti-idiotypic TR antibody on cloned murine T cells was not affected by CS (69). Furthermore, CS did not inhibit the binding of anti-CD3 antibodies which recognize the invariant part of the TR, the CD3 complex (143), as originally suggested by Palacios (119, 120). Furthermore, a single report suggested an inhibitory effect of CS at the level of the TR-associated recognition site of the major histocompatibility complex (118). Based on the present experimental data using a variety of ligands recognizing TR and associated structures there is no evidence that CS affects recognition at the TR level (19, 39).

Alternative pathways of T cell activation, e.g., mediated through binding to the CD2 or CD28 receptor-associated structures, have also been examined; the recognition of both membrane proteins is not affected by CS.

2. Signal Transduction

Biochemical events during lymphocyte activation are schematically summarized in fig. 2 (for review, see refs. 2, 75, and 166). Cell activation can be achieved by direct stimulation of protein kinase C (PK-C) by phorbol ester

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(PE) or by increasing the intracellular free calcium concentration by means of calcium ionophores. The physiological activating signal, the antigen, can be mimicked by anti-TR antibodies or mitogenic lectins. Triggering at the antigen receptor causes increased phosphoinositol turnover, resulting in the release of inositol triphosphate, which mobilizes intracellular calcium stores, and the formation of diacylglycerol, which is the putative endogenous ligand for PK-C. The use of PE and/or calcium ionophores supposedly allows the dissection of the signal transduction pathways and the definition of drug-sensitive events.

a. Phosphoinositol turnover. Activation of T and B cells by anti-receptor antibodies and mitogenic ligands initiates the phosphoinositol turnover, resulting in the formation of diacylglycerol and inositol triphosphate (IP3). CS effects have been studied by various groups (110, 7). CS did not affect activation-dependent phosphoinositol turnover in either T and B cells.

b. Calcium mobilization. The roles of increased membrane permeability and/or intracellular mobilization of calcium stores during cell activation have been investigated (47, 166). Based on recent data a sustained increase of intracellular calcium plays a decisive role in the progression of the cell cycle; a transient increase of intracellular calcium can also be found for nonmitogenic anti-receptor antibodies (89). The effect of CS has been studied by the uptake of $^{45}Ca^{2+}$ (105, 7, 139) and by fluorescent Ca^{2+}-chelating agents such as fura-2 (169, 110). The data obtained in both T and B cells did not show any inhibition of activation-induced calcium uptake and/or calcium mobilization. A single report indicated an inhibition of lectin-induced increases in cytosolic calcium when the T cells were preincubated for 30 min at 1 μg/ml CS (47). The last possibility that a cytosolic calcium increase might be induced by activation was reported for murine lymphocytes (72) and rat mesangial and smooth muscle cells (see section E). Furthermore, activation of the Na^+/H^+ antiport in T cells by Ca^{2+} ionophores but not by PE is CS sensitive (139).

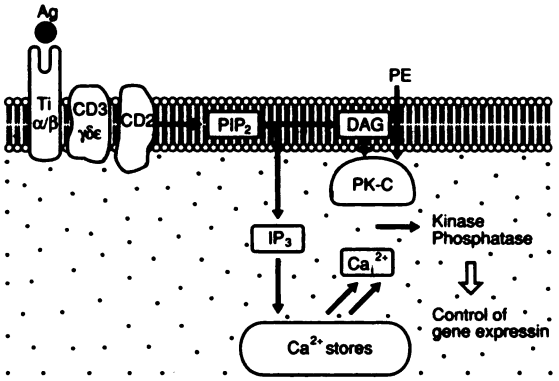

FIG. 2. Early biochemical events of T cell activation. During binding of antigen (Ag) or other mitogenic ligands (antibody, lectins) to the TR complex (Ti, CD3, CD2), phosphatidyl-inositol, biphosphate (PIP_2) are hydrolyzed to inositol-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes internal calcium Ca^{2+} stores, resulting in an increase of intracellular free Ca^{2+} concentration. Diacylglycerol and phorbol ester (PE) activate protein kinase C (PK-C). Subsequent steps include activation of kinases and phosphatases, resulting in phosphorylation and dephosphorylation of proteins transducing the mitogenic signal to the nucleus.

c. PK-C activation. What seemed to be a single entity proved to be a highly heterogeneic family of protein kinases (114, 115). Direct investigations with CS on enzymatic activity of the various members of PK-C has not been performed. Thus, most of the information is circumstantial.

Activation by PE of lymphoid cells was consistently found to be CS resistant (30, 78, 103). However, PE used as the sole activating ligand proved to be a poor stimulator of 50% inhibition of cell proliferation was obtained with peripheral blood lymphocytes between 0.5 to 1 CS μM (B. M. J. Foxwell, unpublished).

TR-mediated activation of PK-C assessed by the translocation of enzyme activity to the membrane in murine lymphoid cells during mitogenic stimulation was CS insensitive (35, 110). The putative substrate of PK-C, the γ- and δ-chain of CD3 (16), are normally phosphorylated after TR-mediated activation in the presence of CS (39). Stimulation of murine L2 cells with concanavalin A results in phosphorylation of proteins between 20 and 200 kD, which is partly inhibited by CS (127). The nature of these phosphoproteins as well as the regulatory events such as altered protein kinase activity or protein phosphatase activity has not yet been investigated.

Activation of B cells results in PK-C translocation to the membrane or nucleus depending on the activating signal (15). So far no published data are available regarding CS effects.

Present experimental data suggest that the block of CS is distal to the calcium and PK-C signals. Later events include increased membrane phospholipid metabolism. Szamel et al. (157, 158) found an inhibition of phospholipid turnover during lymphocyte activation and demonstrated a dose-dependent inhibition of the enzyme lysolecitin acyltransferase. However, inhibition of phospholipase A2 (34) and phospholipid metabolism occurs only at high CS concentrations, which may play a role in the toxic effects of the drug. Increased polyamine metabolism is another feature of cell activation. The rate-limiting enzyme is ornithine decarboxylase in this pathway. Activation-induced ornithine decarboxylase activity was found to be CS sensitive (35).

3. Gene Activation

Although biochemical events in the cytosol and especially within the membrane after T cell activation are well recognized, the links between this so-called second messenger and information transfer to the nucleus are poorly understood. However, because of the rapid progress in molecular research, many genes activated and transcribed during T cell activation have been identified

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(20, 161). The expression of more than 70 molecules is sequentially regulated during this process; only a selected list is given in table 1. The nuclear proteins c-*fos*, nuclear factor (NF)AT-1, c-*myc*, and NFkB are activated immediately after cell activation, followed by interferon-γ, interleukin (IL)-2 between 30 and 60 min, and IL-2 receptor and IL-3 at 2 h. This is only an incomplete list of the complex sequence of the gene activation program (for review, see refs. 20 and 161). Although the temporal order of gene activation may suggest contingent regulatory events, the functional role of most of these molecules is only incompletely established.

CS as a potent inhibitor of cell activation may prove to be a useful tool for the further dissection of regulatory events. Kroenke et al. (84) demonstrated a dose-dependent inhibition of IL-2 messenger RNA (mRNA) in the human T leukemia cell line Jurkat during activation. Furthermore, the synthesis of IL-2 transcripts was also inhibited on isolated nuclei from these cells (84). Similar results were reported for the murine T cell line EL-4; stimulation-induced IL-2 transcripts were not formed in the presence of CS (32). Granelli et al. (53, 52) reported a dose-dependent inhibition of steady state IL-2 mRNA on human peripheral mononuclear leukocytes after different activation signals. As shown in table 1, CS inhibits not only the transcription of IL-2 but a variety of cytokines and protooncogenes. Depending on the mode of the signal pathway (TR, stimulation through accessory differentiation proteins, or through the calcium and/or protein PK-C signal), activation of the genetic program and sensitivity to CS may differ. Under experimental conditions of T cell activation, the antigen in combination with cell/major histocompatibility complex molecules as stimulant is often substituted by antibodies directed to the antigen receptor and other membrane epitopes (CD2, CD28), plant lectins, calcium ionophores, and activators of PK-C. These tools may allow partial dissection of gene activation events, which again may have differential sensitivity to inhibitors.

As an example, activation by anti-CD3 antibody, which recognizes the invariant part of the TR, induces only a very low level of mRNA for IL-4 and almost undetectable levels of IL-2, which led Laing and Weiss (85) to conclude that anti-CD3 stimulation is independent of IL-2 gene activation. However, anti-CD3-induced lymphocyte proliferation is highly sensitive to the inhibitory action of CS. Whether we are confronted with a limiting sensitivity of the technique or whether we are truly looking at different pathways of cell activation can presently not be determined.

Whereas activation of PK-C by PE is CS resistant, activation by calcium ionophore is highly sensitive to the inhibitory effect of CS (39).

Stimulation by a variety of antibodies recognizing epitopes distant from the TR, e.g., CD2, CD28, has been investigated. Stimulation at CD2 has similar sensitivity to CS as TR stimulation. Antibodies against CD28 cause only T cell activation in conjunction with PE or anti-CD3 antibody. An interesting aspect of the CD28-mediated stimulation is the new finding of relative stabilization of cytokine mRNA which usually is short-lived (97). Gene activation through the CD28 pathway was found to be CS resistant (77).

TABLE 1
Summary of proteins induced after T cell activation

Protein	Time	Location*	CS sensitivity	Ref.
c-*fos*	15 min	N	+	136, 137
NFAT-1	20 min	N	–	20
c-*myc*	30 min	N	+	134, 137, 52, 51
c-*src*	60 min	N		44a
NF-kB	30 min	N	?	
γ-Interferon	30 min	S	+	149, 138
IL-2	45 min	S	+	53, 52, 51, 32, 79, 84, 162a
IL-2 receptor	2 h	S	+	51, 52, 45, 135
IL-3	1–2 h	S	+	121, 5, 6
IL-4	>6 h	S	+	85, 84
IL-5	>6 h	S	?	
IL-6	>6 h	S	–	
c-*myb*	16 h	N	?	137
Transferrin receptor	14 h	CM	+	53, 162a

* N, nuclear; C, cytoplasmic; CM, cell membrane; S, secreted.

4. Growth Factor Synthesis

Recent developments resulted in the characterization of the structure and function of factors released during T cell activation. In table 2, the functional properties of various cytokines are summarized. These molecules with molecular weights between 15,000 and 25,000 can be quantified in the cell supernatant using bioassays. Because these molecules have been cloned, information can also be obtained at the transcriptional level (table 2).

An inhibitory effect of CS on mitogen-induced IL-2 synthesis in murine and human lymphocyte and cell lines is well established (84, 32, 53). By contrast, constitutive synthesis of IL-2 by the gibbon leukemia cell line MLA 144 is CS insensitive. Whereas IL-1α, IL-1β, and granulocyte macrophage colony-stimulating factor (GM-CSF) are CS resistant, the synthesis of IL-3, IL-4, and IL-6 and interferon-γ synthesis are inhibited by CS. Effects on other hemopoietic growth and differentiation factors such as G-CSF, M-CSF, IL-7 and IL-8, are not yet reported.

In summary, CS inhibits the synthesis of a broad spectrum of cytokines after cell activation but has no effect on constitutive synthesis and spares hemopoietic growth factor synthesis, e.g., GM-CSF.

5. Growth Factor Receptors

Growth factors act through cellular receptors in an autocrine or synergistic fashion with other factors. The structure and binding properties of a variety of growth factor receptors have been characterized, and the recep-

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TABLE 2
Overview of human cytokines

Cytokine	Molecular weight (thousands)	Cellular source	Biologic activity	CS sensitivity
IL-1α	15–17	Monocytes	Pleiotropic effect on T lymphocytes and fibroblasts (cytokine release, growth)	−
IL-1β	15–17	Monocytes		−
IL-2	14–16	T cells	Growth of activated T cells	+
IL-3	20	Monocytes, T cells	Growth of hemopoietic multipotential stem cells	+
IL-4	20	T cells	Growth factor for T cells	+
IL-5			Differentiation of eosinophils	
IL-6	22–29	Fibroblasts T cells, monocytes	Pleiotropic effects, production of acute phase proteins by hepatocytes, class I expression on fibroblasts	+
IL-7	25		Hemopoietic factor and B cell growth factor	
INF-γ*	40–45	Peripheral blood Leukocytes	Expression of HLA-DR on endothelial cells and fibroblasts; antimicrobial and tumoricidal activity	+
TNF-α†	17	HL-60		+
TNF-β†	25	Peripheral blood		+
GM-CSF	22	Macrophage cell line	Hemopoietic growth factors	±
G-CSF	19	Human bladder carcinoma	Hemopoietic growth factors	?
M-CSF	47–76	Human urine	Hemopoietic growth factors	?

* Interferon.
† Tumor necrosis factor.

tor proteins have been cloned. The relevant findings of cytokine receptors as well as their behavior during T cell activation are summarized in table 3.

a. IL-1R. A membrane receptor protein (M_r 65,000) for murine IL-1α and IL-1β has recently been cloned (155). Transfection of the cloned deoxyribonucleic acid (DNA) into fibroblasts results in the expression of a single class receptor with an affinity of $\sim 5 \times 10^{-9}$ M (K_a). Binding studies, however, of a variety of cells indicated the presence of a second class of receptor (10^{-11} M) (8, 26, 27, 80, 99). The existence for a more complex structure for the multitude of IL-1 effects was anticipated (23). Recent observations suggested major structural difference between the murine IL-1*R* on T and B lymphocytes (10a), which represent separate gene products (17b). Furthermore, the human IL-1*R* has been cloned, which defines two affinity classes of receptor on transfected COS cells (155a). No information is available on up-regulation by mitogenic activation or its own ligand. IL-1 receptor expression and/or function in the presence of CS has not been reported.

b. IL-2R. The complex nature of this receptor has been discussed in excellent reviews (17, 95, 55, 56, 165, 102, 156, 160). Cross-linking studies revealed two receptor proteins, p55 (tac) and p75; both proteins have been cloned (94, 65). For high affinity binding (K_d 10^{-11} M), both receptor proteins have to be expressed. Resting lymphocytes express only very low levels of p55 and p75. Both proteins are up-regulated during stimulation at the antigen receptor or by its own ligand. Defective IL-2*R*,

TABLE 3
Overview of cytokine receptors

Receptor	Molecular weight (kDa)	Binding affinity (K_d) (M^{-1})	cDNA	Upregulation by ligand	CS sensitivity	Ref.
IL-1R	65–100	$\sim 3 \times 10^{-9}$ $\sim 10^{-11}$	+	?	−	8, 23, 26, 27, 80, 70a, 77a, 99, 155, 155a
IL-2R	55	$\sim 10^{-10}$)	+	+	+	96, 65; 160;
	75	$\sim 10^{-10}$) 10^{-11}	+	+	+	165; 135, 109, 52, 51, 47, 46, 25, 42a
IL-3R	140	10^{-10}	+	−	?	76, 122, 112, 48, 76a, 48a
IL-4R	65/70	10^{-10}	−	+	+	116, 117, 123, 42
	110	10^{-8}	+	+	+	24a, 110a
IL-5R	92	10^{-10} 10^{-8}	−			108
IL-6R	80	$\sim 10^{-9}$ $\sim 10^{-11}$	+	?	?	170, 19a

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e.g., expression of only p55 or p75, on tumor cell lines confers only low affinity binding (K_d 10^{-8} M). CS effects on IL-2 receptor have been extensively investigated. Depending on the activating 1igand and the analytical tools, no effect of CS was found on IL-2*R* expression (109, 52, 51, 47) or partial inhibition has been demonstrated (46, 25, 135). Our recent studies on anti-CD3-stimulated human T lymphocyte using both chemical cross-linking techniques and Scatchard analysis indicate that CS inhibits the appearance of IL-2*R* in the 10 nM concentration range (42a). By contrast, lectin (phytohemagglutinin or concanavalin A)-mediated up-regulation of IL-2*R* expression is more resistant (500 nM), data which are in agreement with the studies by Reed et al. (135).

c. IL-3R. Membrane receptor proteins (M_r ~ 140,000) have been reported on murine IL-3-dependent cell lines by chemical cross-linking techniques using ^{125}I-IL-3 (76). Specific binding sites were demonstrated on human monocytes, pre-B, and the myelogenous leukemia cell line kg1. Equilibrium binding studies indicate a single class of high affinity receptors (K_d 10^{-10} M) (48, 122). Interestingly, IL-3 causes a down-regulation of its receptor (112). Effects of CS at the IL-3*R* expression and of function have not yet been performed.

d. IL-4R. Extensive receptor studies on murine lymphocytes identified high affinity binding (K_d 100^{-10} M) and a membrane protein with a molecular weight of 70,000 (116, 123). Recent studies in our laboratory revealed both low and high affinity binding on human lymphocytes and the presence of a trimolecular receptor complex of proteins with molecular weights of 65,000 to 70,000 and 110,000 (42). Binding of IL-4 to its receptor protein is specific and not competed with by any known growth factor including IL-2. During T cell activation by mitogenic ligands or IL-4, all membrane proteins are up-regulated (117). Up-regulation of IL-4R proteins by mitogens and its own ligand is CS sensitive (42b)

e. IL-5R. High affinity binding and a membrane protein with a molecular weight of 92,000 have been identified on human cells (108).

f. IL-6R. The IL-6 receptor protein has recently been cloned (170). Transfection experiments indicated the presence of high and low affinity binding. No data on CS are available.

In summary, CS inhibits T cell activation at more than one site. Whereas signal recognition and signal transduction are not effected, the induction and/or transcription of a few selected genes such as cytokine genes are inhibited. In the absence of cytokine synthesis cells will not proliferate. Inhibition of receptor expression generally occurs at higher CS concentrations. This inhibition may be either direct or due to the absence of the respective ligands, which are known to up-regulate its own receptor proteins (fig. 3).

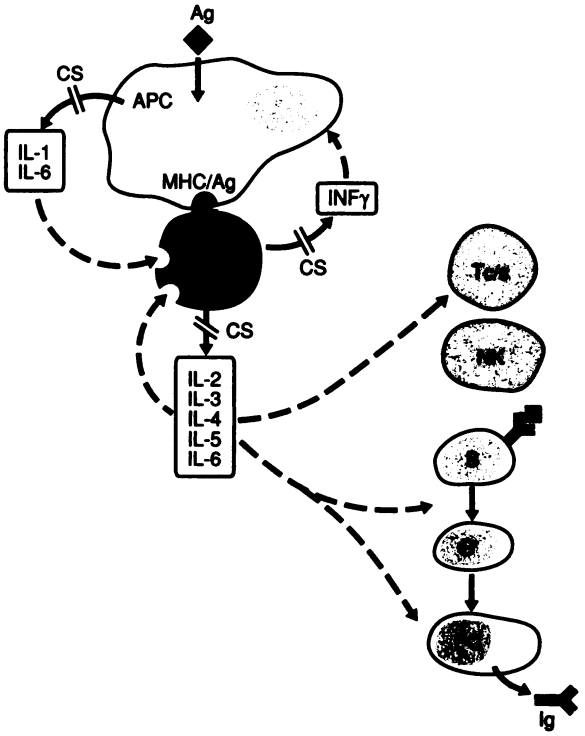

FIG. 3. Cyclosporin effects on an in vitro immune response. CS inhibits the transcription and synthesis of inducible cytokines mainly at the level of the T-helper lymphocyte (T_H). In the absence of these growth factors, the proliferation, differentiation, and maturation of T_H cells and several classes of immunocytes, depending on signals from T_H cells, are functionally arrested. Ag, antigen; APC, antigen-presenting cells; MHC, major histocompatibility complex; Tc/s, cytotoxic and suppressor T lymphocytes; INFγ, interferon-γ; NK, natural killer cell; PC, plasma cell; Ig, immunoglobulin; B, B lymphocyte.

C. Control of Gene Expression

Because CS inhibits induced gene transcription, it may be appropriate to summarize emerging concepts of regulatory events before the discussion of CS effects. A complex pattern of genes is regulated during T cell activation; the activation of cytokine genes plays an important role. Recent investigations of the gene structures and regulatory elements allowed new insights into the physiological control of gene expression.

The general structure of the cytokine gene consists of 4 or 5 exons extending over several kilobases of genomic DNA (59, 161). The genes are single copy, are stable, and do not undergo rearrangements. Chromosomal location of these genes has been established. An intriguing finding is the clustering on the long arm of human chromosome 5, namely, for IL-3, IL-4, IL-5, M-CSF, c-*fms*, and PDGF genes. Another unusual feature is the existence of alternative start sites for the transcription, as has been shown for GM-CSF, IL-6, and IL-2 receptor α-chain (p 55).

Interspecies comparison of the cytokine genes reveals about 80% sequence homology for mouse and human IL-2 and IL-5, whereas mouse and human IL-3 shows less homology.

Because the activation of some cytokine genes is coordinate (table 1), a reasonable postulate would be that

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certain regulatory elements might be common for cytokines such as DNA enhancer sequences as well as inductive mechanisms (63, 161). Several consensus sequences, 8 to 18 nucleotides in length, have been identified in the upstream regulatory regions (the 5′ side of the transcriptional start site) of certain cytokine genes. Such regions located between −250 and −300 base pairs upstream of the start site of IL-2 and IL-2 receptor (α chain) genes have been identified as important regulatory sites. Depending on the activating (mitogenic) ligand a differential gene program can be induced. Thus, separate pathways for the induction of identical genes may exist.

Because of the central role of IL-2 and its receptor in the process of T cell activation, recent investigations have been focused on the regulation of these genes. As for other members of the lymphokine gene family, transcription of the IL-2 gene is rapid and transient (149, 150, 148). Transcription of the IL-2 gene depends on the continued delivery of the inductive signal (167). The half-life of IL-2 mRNA is approximately 1 h. This transience of mRNA is a common feature of many cytokines and especially oncogenes. The 3′-untranslated regions of these transcribed genes carry repeated AU motives that may confer instability to these mRNAs. It is assumed that a degradative RNase system might be induced along with the set of coordinated expressed lymphokines; the activity of the RNase is sensitive to cycloheximide and actinomycin (149, 31). Regulation of the IL-2 gene is mediated at a transcriptional enhancer region located −319 and −52 base pairs 5′ to the transcription initiation site of the gene (45). The functional properties of the enhancer sequences have been tested by the use of reporter genes coupled to ubiquitous expressed promoters. Such a three-part fusion gene is only activated in T cells. As in normal T cells, transfected T cells require both signals, e.g., signals from the antigen receptor and activation of PK-C.

The specific sequences in the IL-2 enhancer necessary for these functions were defined more precisely by internal deletions and base pair mutants transfected in the T cell line Jurkat (29, 45). These investigations identified two DNA sequences, which function as antigen receptor response elements (ARRE), e.g., −285 to −255 (ARRE-2) and −93 to −63 (ARRE-1), and activate a linked promoter in response to a signal from the T cell antigen receptor (fig. 4).

Activation of gene transcription is presumably mediated by binding of regulatory proteins to functional sequences of the IL-2 enhancer. Circumstantial evidence comes from DNase I protection assays with nuclear proteins from activated T lymphocytes (152). Based on competition studies with oligonucleotides from these protected regions, two separate nuclear protein factors can be distinguished binding to the IL-2 enhancer (Fig. 4): the nuclear factor NFIL-2A binding to the proximal regulatory site between −93 and −63 (ARRE-1) and

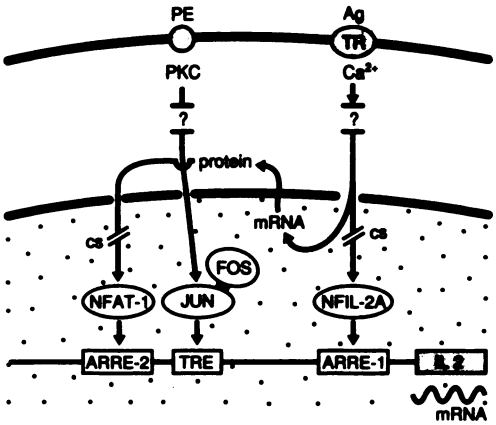

FIG. 4. Pathways involved in the activation of the IL-2 gene and possible sites of CS inhibition (for details see text, modified from Crabtree, 20). Ag, antigen; PKC, protein kinase C; cs, cyclosporine; TRE, tumor promotor response element.

NFAT-1 binding to the distal regulatory site −285 and −255 (ARRE-2) of the human IL-2 enhancer.

The nuclear factor, NFIL-2A, is present in nuclear extracts of resting and activated T cells (29). Despite the constitutive nature of this protein, the −93 to −63 site is hypersensitive to DNase I digestion in nuclear extracts of activated T cells (152). Further evidence for a regulatory role of ARRE-1 is the finding that constructs containing this sequence are suppressible by CS (20). Furthermore, the NFIL-2A-binding site may have a negative function in resting T cells. Thus, this region of the IL-2 promoter may have a dual role, e.g., suppression of the IL-2 promoter in nonactivated cells and activation of its promoter after cell activation.

The other nuclear factor binding to ARRE-2, located −285 and −255 of the IL-2 enhancer, is selectively expressed in activated T cells and thus received the suggestive name NFAT-1, nuclear factor of activated T cells (150). The following evidence indicates that IL-2 gene activation depends on prior formation and/or activation of NFAT-1: (*a*) the appearance of NFAT-1 precedes IL-2 mRNA formation (150); (*b*) delayed addition of protein or RNA synthesis inhibitors after cell activation indicates that NFAT-1 is newly synthesized within 20 min (150); (*c*) deletion of ARRE-2, the binding site for NFAT-1, impairs the activity of the IL-2 enhancer (28, 29); (*d*) nuclear extracts from activated T cells show about 50 times NFAT-1 binding activity as compared to other cell types (150); and (*e*) CS blocks signals from the antigen receptor in constructs containing ARRE-2 (20, 32a). Thus, activation of the IL-2 gene is dependent on the formation of NFAT-1 and binding of the complex to the transcriptional enhancer site ARRE-1. The nuclear factor NFAT-1 has neither been identified nor cloned and nothing is known about its regulation.

Deletion of either ARRE-1 or the ARRE-1 site abolishes most of the IL-2 enhancer activity, which suggests cooperation between the two sites for full activity, al-

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though each protein, NFIL-1A and NFAT-1, may bind independently (29).

Another important regulatory site of transcriptional control is defined by binding of the transactivator protein Tax (Tat-1) of the human T lymphocyte virus-1, which also controls IL-2 and IL-2*R* (M_r 55,000) gene expression (153).

It must be emphasized that most data concerning the IL-2 gene enhancers were obtained in human and murine T cell lines and have to be confirmed on normal T cells.

The present studies on ARRE-1 and ARRE-2 do not explain the requirement of PE for IL-2 gene activation. Activation of PK-C by PE results in the transcription of the c-*jun* oncogene (164, 110a); the protein product JUN binds to the heptameric motive TGACTCA, the AP-1-binding site also known as tumor promotor response element (TRG), which is involved in regulatory control of many genes. An AP-1-binding site is also present in the enhancer of the IL-2 gene at −145 to −158 base pairs (3, 148).[32a] Deletion of the AP-1 site reduces PE-mediated IL-2 gene activation in the murine EL-4 T cell line.

The present view of IL-2 gene activation in the human T cell line Jurkat is shown in fig. 4. At least two distinct pathways, one from the antigen receptor and another by activation of PK-C control IL-2 gene transcription. Based on deletion mutant analysis, the ARRE-1 site most likely plays the primary role for the function of the IL-2 enhancer.

Concomitant with IL-2, the genes encoding the IL-2 receptor proteins are activated, allowing cell differentiation and division. The IL-2 receptor proteins, p55 and p75, which have been cloned (94, 65), are transcribed in a coordinate fashion during activation. Little is known about the control of the p75 chain, whereas the p55 protein is induced by a variety of stimuli such as IL-1, IL-7, PE, and TNF (98, 96, 17a, 126, 90) in addition to signals from the antigen receptor. DNA sequences controlling the IL-2 receptor (M_r 55,000) gene activation are emerging. Binding sites for PE-induced transcription factors e.g., NF-kB and a very similar protein HIVEN 86A have been localized between −255 and −268 of the transcription initiation site (9, 43). NF-kB proteins have also been implicated in IL-2 gene expression (74). Mutation of the NF-kB core sequence in the IL-2 promoter (−206 to −195) partially inhibits mitogen- and human T lymphocyte virus-1 Tax-mediated activation. These data suggest that NF-kB-like proteins have a role in the regulation of both IL-2 and IL-2*R*.

A role for cellular oncogenes (136) in T cell activation is very likely. At least four oncogenes are regulated during T cell activation (table 1). Activation of the c-*fos* and c-*myc* genes, following phytohemagglutinin stimulation by about 15 to 30 min, which are not blocked by protein synthesis inhibitors (136), appeared to represent the primary response. Further evidence for a regulatory role of c-*myc* on cell proliferation was supported by the use of antisense oligonucleotide complementary to the c-*myc* RNA (64, 67). Cells incubated with short antisense oligonucleotides showed delayed or inhibited cell cycle progression as well as the formation of the MYC protein. Although the exact function of c-*myc* and other nuclear oncogenes is unknown, the above data and unpublished studies imply that early oncogenes may contribute to gene activation.

In conjunction with c-*jun*, the c-*fos* oncogene is transcribed after cell activation (110a). The role of JUN binding to the AP-1 site has been discussed above. The FOS protein associated with JUN through the leucin-zipper increases the affinity of the AP-1 protein complex for the AP-1 site (3).

In view of the well-established inhibitory effect of CS on ongoing transcription of induced cytokine genes, recent investigations were aimed at defining the focal point of CS effects at the level of the formation and/or function of nuclear factors binding to cytokine promoters. The first observation of a CS effect was the inhibition of a DNase hypersensitivity site in lymphocytes during activation in the IL-2 gene promoter (152). Investigation of CS on various IL-2 gene promoter constructs did not allow the identification of the exact point of CS action (168). Cotransfection of Tax-1 increased IL-2*R* promoter activity and synergized with mitogenic ligands on IL-1 promoter activity. Cotransfection with Tax abrogated inhibitory effects of CS on the IL-2 promoter (153). Crabtree and coworkers (20, 32a) summarized his investigation of the human T cell line Jurkat (fig. 4) and suggested that CS acts after the synthesis of nuclear proteins; he could not show a direct interference of CS with the binding activity of these proteins. It is evident that our understanding of the physiological control of cytokines and their inhibition by CS is incomplete.

D. Cellular Receptors for CS

1. Cellular Uptake of CS

The initial binding studies with the [^{3}H]CS derivative on whole cells showed specific, saturable, and reversible binding on mouse and human lymphocytes, macrophages, and leukocytes (143, 146). From kinetic and equilibrium binding studies an affinity constant of 10^{-7} M (K_d) was calculated and about 10^6 molecules were bound per cell. Cellular CS binding was found to be independent of active cellular metabolism but temperature dependent. Comparable binding characteristics were found on murine and human lymphoma cell lines (104, 33). However, these authors demonstrated an additional, nonsaturable component of cellular CS binding at high drug concentrations.

LeGrue et al. (92) performed similar whole cell-binding studies with [^{3}H]CS and postulated the existence of low (10^{-7} M) and high (10^{-9} M) affinity-binding sites on blood cells. Low affinity binding of [^{3}H]CS was also found on liposomes (92, 91, 66), which led to the conclusion that

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CS is partitioning in the phospholipid bilayer of the membrane.

Koponen and Loor (83) studied the cellular uptake of dansylated CS in murine cell lines by means of fluorescence microscopy and identified CS in cytosolic vesicles distinct from mitochondria and lysosomes. Dansyl-CS was used in conjunction with flow cytometry to quantify the differential distribution of CS within blood leukocytes. Leukocytes and neutrophils as well as a subset of lymphocytes showed very bright staining (147, 70). Functional analysis of lymphocytes sorted according to the staining property with dansyl-CS revealed an inverse correlation of CS binding with sensitivity to the drug (71).

Monitoring of CS in plasma and whole blood with radioimmunoassay (24, 129, 130) indicated that a substantial fraction of CS is associated with the cellular compartment in the blood (93). Because erythrocytes represent the bulk of blood cells, drug uptake and intracellular binding protein in erythrocytes were investigated (1, 37). The main binding moiety was a protein with a molecular weight of 16,000 to 18,000; the presence of this protein (2×10^5 molecules/erythrocyte) is responsible for the intracellular accumulation of CS. Drug uptake in erythrocytes and a variety of nucleated cells is depicted schematically in fig. 5: at low CS concentration saturable cytosolic binding is observed, whereas at high concentration CS partitions in the membrane, a process which is not saturable (37).

2. *Multiple Molecular Targets of CS?*

Multiple molecular targets were proposed in the membrane, cytosol, and nucleus (table 4).

a. Cell membrane. So far no specific CS receptor protein has been identified in the plasma membrane. However, multiple interactions of CS with other membrane receptors have been reported (table 4). Interference with the IL-1-binding site (4), CD3, and human leukocyte antigen-DR receptor proteins (118–120) has been proposed. Russell et al. (140) demonstrated competition of prolactin binding on whole lymphocytes, but no information is available regarding whether CS competes directly with prolactin for its receptor site in the membrane. Another study of mammary tumor cells showed no inhibition of prolactin binding by CS (100). The functional role of prolactin for T cell activation and proliferation is presently unknown (73, 88).

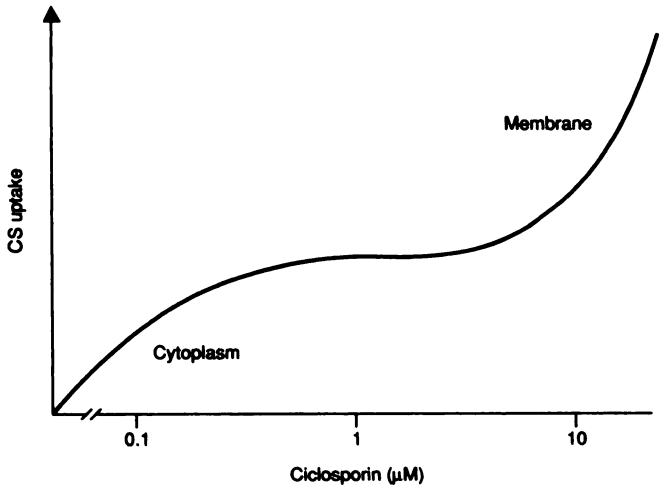

FIG. 5. Cellular [^{3}H]CS uptake. At low concentrations, saturable, cytosolic binding is due to the presence of cyclophilin in the cytosol; at high concentrations, nonsaturable, membrane binding is apparent (37).

TABLE 4
Overview of proposed CS-binding proteins

Cellular localization and target molecule	Affinity	Specificity of binding	Ref.
Membrane			
T cell receptors, MHC class II		?	118, 119
Bile acid transporter		No	171, 172
IL-1*R*		?	4
Lysolecithin acyltransferase		?	157, 158
Prolactin receptor		Yes	140, 73, 88
170-glycoprotein		No	40
Cytosol			
Ornithine decarboxylase		?	35
Calmodulin	10^{-6}	No	18
Cyclophilin	10^{-8}	Yes	60
Nucleus			
Calmodulin		No	154
Cyclophilin		Yes	145
Transcription factors		?	101

Recent studies showed CS binding to the-glycoprotein 170, a membrane protein that is physiologically expressed in kidney and liver cells as well as in multidrug-resistant tumor cells, where it functions as a drug efflux pump (40).

Lymphocyte membrane phospholipid turnover triggered by concanavalin A or calcium ionophore was inhibited at 100 ng/ml CS (157, 158); it was suggested that lysolecithin acyltransferase might be a target of the drug. However, lysolecithin acyltransferase activity was not inhibited in isolated membranes at high CS concentrations (157, 158). Phospholipase A2 was suggested as a possible target of CS, because inhibition of enzyme activity was found at 10 μg/ml (34); however, these concentrations are far beyond pharmacological relevance. By means of [^{3}H]CS-diaziridine photoaffinity labeling, two membrane proteins, e.g., the bile acid transporter in hepatocytes and the glucose carrier in renal tubular cells, were identified as targets of CS (171, 172).

b. Cytosolic molecular targets. In elegant studies, Merker and Handschuhmacher (104) showed uptake of [^{3}H]CS in lymphoma cell lines and identified a protein (M_r 18,000) which bound CS. Further purification led to the detection of the novel CS-binding protein, which received the suggestive name cyclophilin, because it has a reasonable affinity for CS (60, see below). Other cytosolic targets of CS were suggested, e.g., ornithine decarboxylase, hemoglobin, calmodulin, and a phosphoprotein with

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a molecular weight of 45,000. A direct binding and/or effect of CS on ornithine decarboxylase activity was not investigated, and it is assumed that enzyme inhibition is a secondary effect (35). The possibility that hemoglobin in erythrocytes could function as a CS carrier can be discarded, however; commercial hemoglobin preparations may contain variable amounts of the CS-binding protein (M_r 18,000) (37) and led to erroneous conclusions (93). Cell activation depends on the availability of intracellular calcium- and calmodulin-dependent enzyme activation. An attractive hypothesis was formulated that immunosuppression by CS might be mediated through calmodulin. Colombani et al. (18) demonstrated binding of dansyl-CS to calmodulin. CS inhibited the calmodulin-dependent activation of phosphodiesterase. LeGrue et al. (92) examined the CS-calmodulin interaction with the inclusion of inactive CS analogues. Calmodulin binding was demonstrated for both active and inactive CS derivatives. Furthermore, calmodulin-dependent activation of phosphodiesterase, calmodulin-dependent protein kinase activity, or interaction of calmodulin with other acceptor proteins was CS insensitive (92). Foxwell et al. (38, 41) compared the CS-binding properties of purified calmodulin and cyclophilin; specific CS binding could only be demonstrated for cyclophilin; furthermore, cyclophilin bound only immunosuppressive CS analogues (128). In conclusion, present evidence suggests that possible in vivo effects on calmodulin-dependent enzyme activation may be secondary rather than a direct effect of CS. The initial results obtained by Colombani et al. (18) can, however, not be supported by the present experimental findings.

The presence of other CS-binding proteins was further tested by CS affinity gel matrix (37, 38). In addition to the protein with a molecular weight of 18,000 (cyclophilin), a novel phosphoprotein with a molecular weight of 45,000 was identified in the ^{32}P-labeled Jurkat T cell line. The functional significance of this protein is unknown.

c. Nuclear targets. A direct action of CS on nuclear factors controlling gene activation, nuclear oncogenes, or calmodulin has been suggested. Simons et al. (154) reported nuclear lobulation of about 20% of peripheral blood mononuclear cells in the presence of CS. Mahajan and Thompson (101) found a depletion of an RNA polymerase 1 transcription initiation factor in CS-treated lymphosarcoma cells, resulting in the failure to transcribe ribosomal RNA. The significance of this finding is presently unknown. Because most of the nuclear factors show low abundance, the detection of CS binding will be a very difficult analytical task. Presently, no evidence exists for any specific nuclear CS-binding protein.

3. Cyclophilin

The most likely molecular target for CS action is cyclophilin, which has been identified by Handschumacher et al. (60). Cyclophilin is a low molecular weight CS-binding protein (18,000), which has been purified to homogeneity from bovine thymus and human spleen. The amino acid sequence was determined and no homology was found with any other peptide (60, 62), except for the very recent finding of homology with *cis-trans*-isomerase (see below). Two isoforms of human cyclophilin—a major (pI 9.1) and a minor (pI 7.4) form—were separated with nearly identical amino acid compositions (62). The complete sequence of human, rat, and yeast cyclophilin has been deduced from complementary DNA (58, 163); more than 95 sequence homology between human and rat cyclophilin was shown. Southern blot analysis indicated the existence of multiple copies of the cyclophilin gene (58).

The affinity and specificity of the cyclophilin-CS interaction have been investigated. During CS binding, fluorescence emission increase allowed the calculation of a K_a of ~30 nM. Furthermore, binding of CS analogues in this system correlated with their immunosuppressive activity. A similar binding specificity was also obtained by competitive LH-20 column assay (60). Quesniaux et al. (128) examined the specificity of cyclophilin binding using a competitive, solid phase enzyme-linked immunosorbent assay technique. Data from cross-reactivity studies with more than 50 CS analogues with various substitutions on the 11 amino acid indicated that amino acids 1, 2, 10, and 11 are essential for CS-cyclophilin interaction. These amino acids together with residue 3 were shown to be required for CS immunosuppressive activity. Subtle changes in these residues reduced both the affinity for cyclophilin and in vitro immunosuppressive activity (128, 131).

The molecular dynamics and structure of CS binding on cyclophilin were investigated by nuclear magnetic resonance techniques. These studies indicate a compact globular structure of cyclophilin with a hydrophobic core containing 11 aromatic residues (22).

4. Functional Role of Cyclophilin?

The distribution of cyclophilin in lymphoid and nonlymphoid human and animal tissues was investigated. From these studies ubiquitous occurrence of cyclophilin in normal and neoplastic tissues is evident (82, 146). Another intriguing factor is that this protein is not only ubiquitous but also very abundant. Estimates based on enzyme-linked immunosorbent assay measurements are in the range of 5 μg/mg protein (145). Investigations of the subcellular distribution by cell fractionation and immunocytochemistry revealed the occurrence of cyclophilin in both the cytosolic and nuclear compartment (70, 45).

Attempts to define cyclophilin function were undertaken by various groups. An important role of cellular cyclophilin might be the uptake and intracellular concentration of CS in cytosolic and nuclear target sites. Accordingly, cyclophilin would act as a drug-concentrating

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device within the cell. Because of the ubiquitous occurrence of cyclophilin, CS accumulates in all tissues.

Harding and Handschumacher (61) suggested in preliminary experiments protein kinase activity of semipurified cyclophilin. However, these data could not be confirmed with purified material (38).

An exciting new perspective was obtained by the discovery of homology between cyclophilin and peptidyl-prolyl-*cis-trans*-isomerase (PPIase) (36). This recently discovered enzyme in mammalian tissues catalyses the slow *cis-trans*-isomeration of proline peptide bonds in oligopeptides and accelerates the folding of several proteins (86, 87). N-terminal sequence analysis of PPIase indicated identity with cyclophilin. The catalysis of prolyl isomerisation in oligopeptides and of protein folding by PPIase was dose-dependently inhibited by CS. Simultaneously, Takayashi et al. (159) reported that porcine PPI is identical in sequence to bovine cyclophilin and confirmed an inhibitor effect of CS on PPIase activity.

Another exciting finding is the homology between a photoreceptor protein in *Drosophila* and cyclophilin (151); the functional role of these molecules is unknown. Based on these new findings it is proposed that PPIase activity might be involved in T cell activation that is inhibited by CS. CS might thus, by inhibiting proper folding of regulatory peptides, interfere at some level of the activation cascade, e.g., formation of nuclear factors. Specificity of the CS effect on T cells could be explained by selective substrate availability in this compartment.

E. Effects on Nonimmune Cells

1. Antigen-Presenting Cells (including Dendritic Cells)

Antigen uptake and processing was studied in murine and human monocytes and dendritic cells. Whereas Muller et al. (111) showed no effect on antigen uptake and processing, Knight et al. (81) presented evidence for impaired antigen processing. More detailed information concerning this topic may be found elsewhere in this review (Borel, Di Padova).

2. Fibroblasts

Fibroblasts represent an important element in tissue repair and wound healing. CS does not affect these processes (142). An interesting recent comparative study on human fibroblasts and T cell lines has been published (57). Gene activation after stimulation with PE, phytohemaglutinin, and ionomycin alone or in various combinations has been investigated. Whereas inducible genes in the T cell line Jurkat showed variable CS sensitivity, eight inducible genes in the Mrc 5 fibroblast cell line were resistant to CS effects. These studies showed that not only the inciting stimulus but also the cell type is an important factor in the ability of CS to inhibit gene activation.

3. Kidney Cells

Renal dysfunction is a common complication of CS therapy at high doses (162). Functional changes, which consist of reduction of glomerular filtration rate due to preglomerular vasoconstriction, may be associated with morphological changes in afferent arterioles and tubules (107). In the arterioles, degeneration and necrosis of smooth muscle and endothelial cells and thrombosis and obliteration of the lumen may be observed; proximal tubular cells may show vacuolar degeneration, inclusion bodies, and calcification. To discern the biological events resulting in toxicity, CS effects were investigated in tubular cell of canine (MDCK) and porcine (PK1) origin. CS at ≥ 10 μM inhibited protein synthesis and proliferation; ultrastructural investigations of CS-treated cells revealed vacuolisation, lipid deposition, and increased numbers of phagolysosomes resulting in cell death (145). Similar results were obtained with primary cell cultures.

Receptor-mediated mobilization of intracellular free calcium was investigated in smooth muscle and mesangial cells (124, 125, 106). An angiotensin II-induced increase in cytosolic free calcium, as measured with quin 2, was enhanced by CS. Furthermore, CS increased basal $^{45}Ca^{2+}$ influx and augmented angiotensin II-stimulated influx and efflux of calcium from smooth muscle and mesangial cells. Furthermore, vasopressin-induced mesangial cell contraction was enhanced by CS. These results suggest that CS increases the membrane permeability for calcium and also augments receptor-mediated increases in cytosolic free calcium and cell contraction.

4. Hepatocytes

Investigations of primary rat hepatocyte cultures indicated that CS inhibits bile acid transport (10). Recent investigations of liver mitochondria suggested that CS affects the permeability of the inner mitochondrial membrane (14). Increased receptor-mediated calcium uptake has also been shown for primary hepatocytes (113).

5. Hemopoietic cells

A distinctive feature of CS immunosuppression is the absence of effects on the hemopoietic tissue (11–13, 141). In vitro studies of bone marrow cells confirmed the in vivo findings. The formation of CFU-C and BFU-E colonies was only inhibited at toxic (≥ 10 μg/ml) CS concentrations, whereas T cell colonies were more sensitive (50, 68). Recent experiments suggested that CS at 1 μg/ml even enhances the formation of CFU-C and BFU-E in vitro (44) and after bone marrow transplantation (132). Present data clearly show the absence of any hemopoietic side effects.

F. Critical Comments

1. Extrapolation from in Vitro Systems to the Situation in Vivo

Data obtained in vitro may give interesting information about some aspects of cell activation but certainly

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will never reflect the complexity of an in vivo system. Thus, conclusions drawn from in vitro studies cannot directly be extrapolated to in vivo situations. Furthermore, what seems relatively simple and easily controlled in in vitro culture systems depends on a variety of variables that have to be considered.

2. *Variables of Cell Culture*

a. Culture medium. The choice of the medium and especially the various additions, such as serum, antibiotics, or mercaptoethanol, may influence the results. Among these factors the source and final concentration of serum play the predominant role. To avoid the variability of different serum batches, many investigators use serum-free culture conditions.

b. Choice of the stimulating ligand. Lymphocytes may be activated by antigens (tetanus toxoid, *Candida*, etc.), antibody against TR structures, mitogenic lectins, PE, calcium ionophores, only to mention a few. Stimulation by these various cell activators induce a differential genetic program which has also differential sensitivity to inhibitors.

c. Concentration of cell activators. Increasing the concentration of mitogenic lectins result in in vitro immune responses that are more resistant to pharmacological inhibitors; the same may be true for the addition of exogenous growth factors; thus, careful studies correlating concentrations of the stimulating ligand with the response are important for pharmacological studies.

d. Cell population. The composition of human peripheral mononuclear leukocytes is an important factor; many techniques exist to deplete T and/or B cells and antigen-presenting cells; the relative enrichment or absence of a given cell population demands careful phenotypic analysis; not only the purity of the cell population but also techniques applied to achieve the identical purity may have an influence on the experimental results.

e. Cell density. Cell-cell interactions are very important in this in vitro culture system and thus the cell density plays an important role.

f. Duration of culture. The possibility that a pharmacological agent may delay an optimal response has to be checked by analyzing the cell culture at later time points. Based on these considerations it may be important to test a given pharmacological agent at various cell culture conditions, e.g., different mode of cell activation, variable concentration of mitogenic ligand variation of cell density, etc.

3. *Pharmacological Dose-Response Curves and Comparison with Other Immunosuppressants*

Recent investigations of the mode of action of CS were performed using single drug concentrations; to characterize inhibited biological response by a pharmacologic agent, a careful dose-response curve has to be established, e.g., 50% inhibition of response and determination of the concentration resulting in a full inhibition is achieved. A comparison with agents having similar pharmacological profile under the same experimental conditions is important. Direct comparison of results is only possible when the results are presented in molar ratios. Recent investigations by Tocci et al (162a) and Dumont et al (27b) compared directly the immunosuppressive effects of the macrolide FK506 with CS. Both immunosuppressants inhibit a similar if not identical set of induced lymphoid genes, but on a molar basis FK506 is 100 times more potent than CS (162a, 27b).

4. *Primary Cell Cultures Versus Clones and Cell Lines*

Primary cell cultures from human peripheral mononuclear leukocytes show some disadvantage because of the variable composition of the cells and interindividual variations. Clones and cell lines have many advantages in terms of culture but may not reflect the normal cell control. Many data regarding CS were obtained from cloned cells and cell lines and thus have to be confirmed in primary culture systems.

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Errata

In the article "What Is Synergy?" by M. C. Berenbaum (Pharmacological Reviews **41:** 93–141, 1989), the following corrections should be noted. Page (p.) 96, paragraph (¶) 4, sentence (sent.) 6: $f(d_a,d_b)$; [**not** $f(d_{a,b})$]; p. 98, ¶ 2, sent. 1: $S(d_b)$ [**not** $S(d_{bb})$]; p. 105, ¶ 3, sent. 2: the dose of B (**not** 8); p. 105, ¶ 3, sent. 3: suppose f_a is the (**not** f_a the); p. 111, equation 29:

$$\frac{d_a}{M_a\left[\frac{E}{E_{\max}-E}\right]^{\frac{1}{M_a}}}+\frac{d_b}{M_b\left[\frac{E}{E_{\max}-E}\right]^{\frac{1}{M_b}}};$$

p. 112, ¶ 1, sent 5: 21 of C (**not** $2I$ of C); p. 121, legend Fig. 18; sent 3: $2M_a\ M_b$ (**not** $^2M_a\ M_b$); p. 121, equation 38: $S(d_a, d_b) = e^{-\beta(d_a n\ +\ d_b n)}$; p. 126, legend Fig. 22, sent. 3: $I^{-1}{}_{4_0}$; p. 126, ¶ 3 sent. 2: surface modeling (**not** methods); p.131, ref. 31: F2α. (**not** F2); p. 13, ref. 266: ISIS-2 (**not** 1515-2); p. 137, ref. 364: L3T4$^-$Lyt-2$^-$ (**not** L3T4-Lyt2); p. 138, ref. 414: Popik (**not** Potpik).

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