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Pharmacology of Cyclosporine (Sandimmune)

V. Pharmacological Effects on Immune Function: In Vitro Studies

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A. Introduction

CYCLOSPORINE (CS)* is an innovative immunosuppressant agent, which specifically and profoundly affects the immune response to foreign cells and molecules and which differs from other immunosuppressive drugs in several aspects (28). In particular CS, at pharmacologic doses, is not a cytotoxic agent and it is selective and specific in its immunosuppressive activity. Its action is mainly restricted to cells of the lymphoid lineages, T cells, and B cells (284, 392), and it affects other systems such as the hematopoietic system only partially or indirectly. According to the prevailing view and to several in vitro studies, CS exerts its effect by blocking the activation of lymphocytes at an early stage. CS inhibits lymphokine secretion and other T cell functions, but spares effector functions of activated cytotoxic T lymphocytes (CTL) and allows the differentiation of alloantigen-activated suppressor T (Ts) cells (151). Different subpopulations of T cells (151) and B cells (68) have different susceptibility to the drug. Its inhibitory activity is reversible as soon as the drug is washed away from the cultures. CS action is primarily the result of intracellular events.

An immune response can be divided into several overlapping phases: antigen encounter, recognition, activation, amplification, and regulation (294). Several in vitro systems have been developed to assess the distinct mechanisms of intercellular communication and the different cells which mediate effector functions. The antigen represents only the initial trigger. The later development of the immune response seems mainly linked to an autonomous and complex regulatory system which greatly depends on the release of and the response to soluble mediators. Accessory cells mediate the encounter with foreign antigens. The recognition phase involves, at the cell level, the strict interaction among accessory cells, T cells, and eventually B cells and, at the molecular level, the recognition by the T cell receptors (TCR-α/β or TCR-γ/δ) of antigenic fragments in association with major histocompatibility complex (MHC) class I or class II gene products (23, 40). The TCR apparently interacts via its variable elements (Vβ, Dβ, Jβ, Vα, and Jα) with the complex formed by antigen and MHC. This event is converted into an activation signal inside the T cell. Other extracellular events such as interleukin (IL)-1 and accessory molecules on the surface of the interacting cells [lymphocyte function-associated molecule 3 (LFA-3) on the accessory cell and CD2 on T cell] also contribute to signaling (79).

Subsequently, cell activation, proliferation, and differentiation as well as the recruitment of other cells (T cells, B cells, etc.) ensue. Cell communication is linked to cell interactions through specific receptors on the activated cells and the release of soluble mediators called lymphokines (more than 15 active molecules have been cloned). The release and regulation of lymphokines represent critical events of in vitro and in vivo responses. Activated T cells secrete a variety of lymphokines, which stimulate macrophages and several other T and B cell types, and hematopoietic colony-stimulating factors, which activate bone marrow precursors of granulocytes, macrophages, eosinophils, and mast cells and which stimulate these cells to greater functional capacity. In vivo the recruitment of other cell types and amplification systems (complement, coagulation system, etc) occurs. In this way, T cells aid inflammation. The linkage between specific and nonspecific events in the immune response is essential for the deployment of the immunological message.

Several in vitro studies have localized the events affected by CS in the early phase of the immune response, mainly at stages involving antigen presentation by the accessory cells and T cell activation. It has been suggested that CS acts on T cells by blocking the TCR-α/β (308–310), by inhibiting the expression of receptors for IL-1 and IL-2 (12, 37, 70, 221), by interfering with the prolactin receptor (156, 344), by intercalating into the cell membranes (230), by changing membrane dynamics (254), by interfering with plasma membrane phospholipid metabolism (374, 379) or with the activation of

*Abbreviations used are: CS, cyclosporine; CTL, cytotoxic T lymphocytes; Th, helper T; Ts, suppressor T; TCR, T cell receptor; IL, interleukin; ALS, horse anti-human lymphocyte serum; IFN, interferon; NK, natural killer; PHA, phytohemagglutinin; Con a, concanavalin A; MLR, mixed lymphocyte reaction; EBV, Epstein-Barr virus; PWM, pokeweed mitogen; LPS, lipopolysaccharide; MAb, monoclonal antibody; mRNA, messenger ribonucleic acid; HLA, human leukocyte antigen; CML, cell-mediated lympholysis; TNF, tumor necrosis factor; LFA, lymphocyte function-associated molecule; LAK, lymphokine-activated killer; APC, antigen-presenting cell; Ig, immunoglobulin; TI, T cell-independent antigen; TD, T cell-dependent antigen; SAC, *Staphylococcus aureus* Cowan strain; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; PDB, phorbol 12,13-dibutyrate; GM-CSF, granulocyte macrophage colony-stimulating factor.

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phospholipase A2 (86), by causing cell depolarization (106, 292), by inhibiting secretion of lymphokines (37, 91, 152, 154), and/or by favoring the induction of Ts cells (414). By now many of these hypotheses are no longer valid. Preincubation with CS prior to T cell activation has no immunosuppressive activity in vitro (151, 325), and this argues against a close association of CS to the plasma membrane, because CS must be present along with the relevant antigen/mitogen to exert its biological function. CS exerts only a minor disorganizing effect on lipid membranes (299), and high doses of CS (100 μg for 2×10^7 mouse lymphocytes) are required to observe an increase in the rigidity of the plasma membrane (254). In addition, a specific cell membrane lipid receptor for CS has not been found (230, 357), and other studies have shown that CS does not block signaling at the cell surface. CS does not directly interact in a competitive manner with cell surface receptors such as the TCR-α/β or preformed IL-2*R* (53, 186, 302, 347). On the other hand contradictory results exist on specific binding of CS to prolactin receptors (156, 244, 344). Moreover, CS inhibits the response of some genes but not of others, and it does not impede the basic machinery of cellular activation and proliferation.

At present, accumulating evidence suggests a cytoplasmic and/or nuclear site of action at pharmacological doses and a selective inhibition of lymphokine gene transcription (5, 54, 191, 360). On the other hand, CS does not affect the synthesis of virally induced interferon (IFN)-α or IFN-β by fibroblasts and lymphocytes (1, 178).

A persistently controversial issue is the capacity of CS to interfere with the expression on stimulated T cells of low and high affinity IL-2*R* (25, 37, 325, 330).

As already mentioned, lymphokines have an important role along the pathway to immune function. Secretion of many lymphokines such as IL-2, IL-3, GM-CSF, and IFN-γ is easily detected within 24 h of stimulation with mitogen or antigen (55). Lymphokines are not lineage specific and regulate the activation, proliferation, and differentiation of macrophages, T cells, B cells, and a variety of other cell types and the development of effector cells. The inhibition of lymphokine release blocks most T cell-dependent events such as T cell clonal expansion, the activation of CTL, macrophages, and other cells [e.g., B cells and natural killer (NK) cells]. All known lymphokines have effects on more than one cell type, and some, such as IFN-γ, IL-1, IL-2, IL-4, IL-5, and IL-6, have multiple effects on a variety of immunocompetent cells. A complex picture emerges, in which each lymphokine has pleiotropic effects on multiple subsets of lymphoid cells and in which most cell types respond to more than one lymphokine. Lymphokines are secreted mainly by Th cell subsets, but some of them are secreted by B cells, macrophages, and fibroblasts.

However, the inhibition of cytokine gene expression by CS does not explain all of its biological effects, and alternative hypotheses have been suggested. One of these assumes that the block of the immune response occurs at the level of accessory cells. Thus, inhibition of lymphokine production would be the result of the lack of activating signals normally delivered to T cells by antigen presented on accessory cells (247, 312, 403).

Another important issue is the possibility to distinguish in vitro between pharmacologic cytostatic and cytotoxic doses. Above a certain concentration CS exhibits in vitro a nonspecific cytostatic effect on many cell types. This cytostatic effect is readily reversible (hours) after CS withdrawal. Concentrations of CS above 0.5 to 1 μg/ml are already cytostatic (155, 279), whereas concentrations of 5 to 10 μg/ml are potentially toxic to T cell clones or other cells in vitro (103, 240, 302). Differences in sensitivity of various cells and of different cell functions have been described (279). CS can inhibit DNA synthesis and proliferation of keratinocytes without affecting other biological processes such as the expression of human leukocyte antigen (HLA)-DR antigens induced by IFN-γ (103).

The concentration of drug attained in vivo represents an important parameter to define the active pharmacological range and to differentiate specific and toxic effects. Differences in pharmacologically active levels of the drug between man and rodents have been reported. Selection of the appropriate CS dose for in vitro studies is complicated in humans by marked inter- and intraindividual variability in drug pharmacokinetics and pharmacodynamics such as variations in drug absorption, volume of distribution, and metabolism. When administered systemically, CS is well distributed in almost all tissues, but, in general, the concentration of CS is higher in some tissues (289). In humans, Ried et al. (337) and Duell et al. (74) reported that CS concentration in skin was 1.72 μg/g tissue when the serum level was 0.2 μg/ml and 1.9 $\pm$ 0.4 (mean $\pm$ SE) μg/g tissue with serum levels of 0.25 $\pm$ 0.08 μg/ml. However, the important parameter is the active fraction of CS which is difficult to define (73). Studies have suggested that murine blood levels of >1 μg/ml need to be maintained to achieve inhibition of transplantation rejection (7). In another study, blood levels of CS in the order of 3 μg/ml in mice were necessary to obtain immunosuppression (368). In humans, the putative therapeutic level in serum is normally lower and has been set at 0.1 to 0.25 μg/ml. In man, whole blood trough levels of 0.35 to 0.45 μg/ml in the first 6 wk posttransplantation and 0.15 to 0.25 μg/ml thereafter have been recommended for transplant recipients (195). In addition, different methods have been used to measure CS levels in whole blood or serum, and their results often do not correlate (for more details see section III). In conclusion, these data prevent a direct comparison of the effects of CS in vivo and in vitro because the concentration of CS available in the tissues is not easily defined.

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Another ambiguity derives from the suggestion that CS metabolites 17 or 1 might contribute to the immunosuppression in renal transplant recipients (209). In this case, the correlation between in vitro and in vivo levels may become even more complex.

Furthermore, the reversibility of CS action complicates ex vivo studies. The washing of blood cells in the laboratory readily removes the drug, and in the absence of CS the functions of peripheral blood mononuclear cells (PBMC) return rapidly to normal. CS treatment does not influence the cytotoxic capacity of PBMC as measured in cell-mediated lympholysis (CML), antibody-dependent cell cytotoxicity, and NK (405, 409). In human patients treated with CS, tests of NK cells and proliferative responses to phytohemagglutinin (PHA), concanavalin (Con A), alloantigens, IL-2, tetanus, and *Candida* antigens are often reported as normal (85, 405), even if impairment of NK activity in transplant recipients was described (128). In addition, several studies show no change in the proportion of T cell subsets, including CD4 and CD8 subsets, during prolonged CS administration (85, 313, 405). However, a decrease of IL-2*R* (Tac)-positive cells in the peripheral blood of patients with multiple sclerosis after CS treatment has been found (41). The capacity of PBMC of patients receiving transplants to retain normal lymphokine production is controversial. Although some authors find that IL-2 secretion after PHA stimulation is normal (405), other studies show a decrease of IL-2 and IFN-γ in the early posttransplantation period, with no change in lymphotoxin production (76, 128, 257). Also, Yoshimura and Kahan (442) reported reduced IL-1, IL-2, and IFN-γ generation by PBMC after human kidney transplantation.

Until now much of the research on CS has focused on the influence of this drug on the production of IL-2 and IL-2*R* expression. Few studies have analyzed the effect of CS on other newly defined aspects of the immune response. A review of some of these studies suggests that CS may actually interfere with other intercellular circuits presently not considered. Among human $CD4^+$ T cells, functionally distinct helper, cytotoxic, suppressor, and helper- and suppressor-inducer subsets exist (179, 274, 406, 441), even if in some cases their demonstration at the clonal level is still lacking. In the mouse, four distinct $CD4^+$ ($Lyt\text{-}1^+$, $L3T4^+$, $Lyt\text{-}2^-$) Th cell subsets have been described (136). In the mouse, $CD4^+$ T cell clones can be grouped in two types, differing both in function and lymphokine secretion (214, 278). The first type of Th cell, designated Th1 or T inflammatory cell, synthesizes IL-2, IFN-γ, and lymphotoxin [tumor necrosis factor (TNF)-β] but not IL-4; the second type of Th cell (Th2 or T regulatory cell) synthesizes IL-4 and IL-5 but not IFN-γ or IL-2. Both types of Th cell synthesize IL-3 and GM-CSF (49, 278). Lymphokines having synergistic actions, such as IFN-γ and lymphotoxin or IL-4 and IL-5, are often produced by the same T cell type, whereas the antagonistic lymphokines IFN-γ and IL-4 are produced by different cells. IL-2 and IL-4 are, respectively, the autocrine growth factors for IL-2-secreting cells and Th2 cells, even if both cell types respond to IL-2 (89). These distinct patterns of lymphokine synthesis correlate with function. Th1 cells mediate delayed type hypersensitivity, kill target cells in an MHC class II-restricted manner possibly as a result of the production of TNF-α and TNF-β (391, 435), suppress specific antibody responses driven by cloned helper lines, and help polyclonal but not specific B cell responses (193). Th2 cells provide help for IgE synthesis, for specific and polyclonal antibody secretion by B cells, and for differentiation of cytotoxic T cells. Similar Th cell subsets have been found in vivo (377). Th1 cells are long-lived recirculating cells which readily respond to stimulation by lymphokine secretion. Th2 cells are precursors that require priming before lymphokine secretion. It has been hypothesized that Th1 are available early in the response and secrete IL-2 and IFN-γ directly in response to stimulation with antigen on accessory cells. Th2 cells would arise only later in the response induced by a different set of signals and would secrete IL-4 and IL-5 (377). A functional heterogeneity has been described also within human $CD4^+$ T cell clones, although a clear distinction in Th1 and Th2 subsets has not yet been proven (343, 400). In the human system, $CD4^+$ T cells with cytotoxic capacity release more IL-2, IFN-γ, and TNF-β. A point of disagreement is the production of IL-4, which is produced by both types of human $CD4^+$ clones (343). The main function of the noncytotoxic T cells is to provide help for B cell differentiation, whereas the cytotoxic T cells are the most important effector cells in inflammatory reactions and delayed type hypersensitivity.

Even if a rigid distinction in Th1 and TH2 cell subsets may not be correct (435), it is evident that heterogeneity in the Th populations is present and that these subsets have different requirements in terms of antigen-presenting cells (APCs) and pattern of lymphokine secretion (136, 190).

Recently, in the murine system, two other subsets of T cells, distinguished by a different TCR (TCR-γ/δ), have been reported (111). The first T cell subset is $CD4^-8^-$ and is found as a minor population in the thymus, spleen, or peripheral blood or in the skin as dendritic epidermal cells (111). The second TCR-γ/δ-expressing T cell is $Thy\text{-}1^-$ and $CD8^+$ and is found among the intraepithelial lymphocytes of the small intestine (229). Information on accessory cell requirements and lymphokine secretion and responsiveness are under investigation, and the immunosuppressive effect of CS on these newly defined T cell subsets is still unclear.

The action of CS on these newly defined intercellular circuits and T cell subsets may be particularly important also to define the effect of CS in vitro and to interpret the effect of CS in vivo. Recent data suggest that CS

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may not act in vivo as it does in vitro. It was shown in a mouse model that CS failed to inhibit the appearance of IL-2-secreting T cells and CTL following the injection of allogeneic spleen cells in the footpad (200). This study raises questions about the mechanism of action of CS as studied in vitro.

B. Accessory Cells

1. Introductory Remarks

Peripheral blood monocytes, tissue macrophages, fixed tissue histiocytes, dendritic cells, Langerhans' cells, B cells, and occasionally other cell types such as endothelial cells constitute a heterogeneous group of cells that play a central role in the presentation of antigen to specific T cells. In addition, most APCs fulfill an "accessory function" for T lymphocytes because they produce a variety of soluble factors involved in host defense and inflammation and carry out a fundamental protective function by ingesting and killing invading organisms.

In general, presentation of antigen fragments by APCs and the production of soluble mediators are required for the generation of an adequate immune response. Antigen-presenting cells, in general, are a source of cytokines such as IL-1, TNF, transforming growth factor, and IL-6 (320, 401). Moreover, during cell to cell contact they may help T cell activation through additional ligand receptor interactions (e.g., LFA-3/CD2 interaction) (79). The direct or indirect effect of CS on several basic functions of the macrophage and on antigen processing and presentation by accessory cells is still under intense investigation.

2. Macrophage Functions

In vitro CS exerts only marginal effects on many basic functions of accessory cells; in vivo data confirm that probably this cell is not a main direct target of CS action. However, CS induces indirect effects, because many of the activities of the macrophages, including phagocytosis, migration, chemotaxis, and release of procoagulant activity (PCA) are dependent on T cell-derived lymphokines. In general, these functions remain intact as long as lymphokines are present, regardless of the concomitant presence of CS. Only a few studies have shown opposite data.

a. Activation, phagocytosis, and chemotaxis. The phagocytosis of bacteria and *Candida albicans* (72, 192, 303), the blood clearance of colloidal carbon by Kuppfer cells (256), the cell migration in response to some chemoattractants (C5a) or lymphokines (72, 73, 192, 388, 429), and the secretion of plasminogen activator and lysozyme (429) are not directly influenced by CS (table 1). On the other hand, CS causes in vitro an inhibition of chemotaxis toward formylmethionyl-leucyl-phenylalanine in pulmonary alveolar macrophages (72, 73) and an inhibition of superoxide release by pulmonary alveolar macrophages (72, 73). In vivo, with the exception of chemotaxis, the effect of pharmacological amounts of CS on pulmonary alveolar macrophages is modest (73). On the other hand, CS in vitro can inhibit, in a dose-dependent fashion, the activation of rodent macrophages to the tumoricidal state by macrophage activating factor plus lipopolysaccharide (LPS) (437).

b. Prostaglandin (PG) synthesis. CS has no major direct effect on PG synthesis even if it has been reported that CS (0.5 μg/ml or more) causes an increase in PGE_2 synthesis in macrophages (82, 83, 354, 424) and in somatic cells (236). The levels of PGE_2 induced by CS are considerably lower than those induced by LPS or phorbol 12-myristate 13-acetate (PMA) (424), but already at these levels PGE_2 has an immunosuppressive function similar to that of CS, because it suppresses T cell activation, blocks IL-2 production by activated lymphocytes, decreases the expression of class II MHC antigens by macrophages and the production of biologically active TNF and IL-1, and facilitates the activation of Ts cells (112, 205, 206, 328, 378). It has also been suggested that an enhanced secretion of PGE_2 might be involved in the modulation of antigen presentation by monocytes (83). Two monocyte subsets (small and large monocytes) with different sensitivity to CS have been described. CS enhances PGE_2 production in small monocytes at concentrations of 0.5 μg/ml, whereas large monocytes do not respond until the dose of CS reaches 2.0 μg/ml (83). However, the addition of indomethacin in vitro does not abolish the action of CS, and PGE is detectable in culture supernatants only several hours after the addition of CS and only when relatively high doses of CS (>0.5 μg/ml) are used (83, 354, 424). These data suggest that prostanoids are not the cause of the immunosuppressive effect of CS. Furthermore, the effect of CS on PGE production by macrophages seems to be mediated through the primary effect of CS on T cell lymphokine release (129). It is known that several cytokines can regulate eicosanoid release; IL-1 induces the release of PG from multiple cell types, whereas IFN-γ (10 units/ml) can completely inhibit LPS-induced PGE_2 release from monocytes (32). Therefore, a block of IFN-γ release mediated by CS could indirectly enhance the secretion of PGE_2. This fine regulation and interaction among PG, monokines, lymphokines, and other factors may be important to understand certain effects of CS. In addition, some studies suggest that monocytes can be activated to two states, one characterized by high levels of PG release and a second one in which PG release is blocked (32). The effect of CS on these activated populations is not known, but such a study might provide additional useful information. CS markedly suppresses, in a dose-dependent manner, the release of PGE_2 in lymphocytes (0.08 μg/ml CS) and slightly in neutrophils (0.8 μg/ml) (292).

Other arachidonate metabolites, thromboxane and prostacyclin, are also affected by CS (354). Thromboxane is a vasocostrictor and a promoter of platelet aggregation

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TABLE 1

Major functions of mononuclear phagocytes and their modification by cyclosporine

Function	Species	Effect of CS	CS concentration in vitro (μg/ml)*	CS dose in vivo (mg/kg)	Reference no.
Chemotactic migration to					
Casein	Man	=	5		192
	Mouse	=	ND		429
FMLP	Rat	−	0.001–1		73
FMLP	Rat	−		5–15 i.p.	72
C5a-activated serum	Rat	=		5–15 i.p.	72
	Guinea pig	=	5		388
Phagocytosis of					
Staphylococci	Man	=	1		303
Candida	Man	=	5		192
Colloidal carbon	Mouse	=		200 p.o.	256
Latex beads	Mouse	=	<50		337a
S. aureus	Rat	=		5–15 i.p.	72
Secretion of					
Lysozyme	Mouse	=	ND		429
PG	Man	+	1		354
	Man	+	0.5		424
	Man	+	ND		82
	Man	+	0.5–1		83
Acetyl glycosaminidase	Mouse	=	ND		429
Plasminogen activator	Mouse	=	ND		429
PCA (thromboplastin)	Man	−	0.1		140
	Man	=	0.5–50		44
	Man	+	5		43
	Guinea pig	−	0.5		388
	Man	=	0.1		309
IL-1	Mouse	−	0.05		37
	Murine cell line	−	0.1		5
	Mouse	=	0.5		38
IL-6	Man	=	10		Personal observation
	Mouse	=	10		Personal observation
TNF-α/cachectin	Man	=	1		84
Superoxide release					
	Rat	−	0.01		73
	Rat	=		5–15 i.p.	72

* +, increased; −, decreased; =, unchanged; ND, no data available.

and is increased by CS (83), whereas prostacyclin which counteracts the action of thromboxane is inhibited in a dose-related way (86, 354). CS (1 to 30 μg/ml) does not inhibit cyclooxigenase activity in this system but seems to cause inhibition of phospholipase A_2 (86). Thus, it is possible that CS causes an imbalance between the synthesis of prostanoids.

c. PCA. Activated monocytes secrete a PCA (thromboplastin) which induces clot formation in platelet-poor citrated human plasma. The generation of PCA is dependent upon interaction between monocytes and activated T cells (90). The effect of CS on PCA is still uncertain. Whereas in some studies CS inhibited this activity (140, 388), in others CS increased PCA (417) or had no effect (44). The effect observed by Helin and Edginton (140) and Thomson et al. (388) may be indirect and linked to the inhibition of lymphokine production by T cells (90, 140). TNF stimulates monocyte PCA (44).

CS, at high concentrations (5 μg/ml), enhances the synthesis and release of PCA and factor VII induced by LPS, PHA, IL-1, and TNF (43, 45). The high doses of CS required and the concomitant requirement for IL-1 and TNF suggest that the relevance of this phenomenon for the formation of fibrin thrombi in transplanted organs should be limited, as suggested also by clinical studies (126).

d. Monokines. CS does not inhibit the capacity of normal macrophages to release IL-1 (152, 154) but suppresses IL-1 production by macrophage cell lines (309). Even if in earlier studies Bunjes et al. (37) and Andrus and Lafferty (5) described a block of IL-1 secretion by macrophages after CS treatment, in later reports it was shown that CS does not directly inhibit the release of IL-1 by accessory cells (152, 154) and that the reduction in IL-1 secretion is mediated through an effect of CS on T cells (3, 38). CS does not reduce the levels of IL-1α

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and IL-1β messenger ribonucleic acids (mRNAs), induced by Con A plus PMA (120).

A positive regulation of IL-1 production by monocytes through secretion of CSF (272) or IFN-γ by T cells (6, 443) is established. A likely mechanism to explain the decreased synthesis of IL-1 by macrophages is a reduction of IFN-γ production by activated T cells, because the production and gene expression of TNF and IL-1 by elicited murine peritoneal macrophages is up-regulated by IFN-γ (52). TNF may also be involved in this loop of activation. $CD4^+$ T cells induce membrane IL-1 on macrophages either by direct contact during antigen presentation or by releasing lymphokines such as TNF (416).

A block of IL-1 secretion would not explain the defect of antigen presentation by macrophages, suggested by some authors as a possible mechanism of action of CS, because the addition of IL-1 in vitro does not reverse the defect (82, 312, 367).

The synthesis of TNF-α, another cytokine synthesized by macrophages, is not directly inhibited by CS (84). PGE_2 and IFN-γ appear to have opposing effects on the synthesis of TNF by macrophages stimulated with LPS. The inhibitory effect of PGE_2 is blocked by IFN-γ treatment (305).

IL-6 secretion by resident murine peritoneal macrophages and by human PBMC stimulated with LPS is not affected by CS at doses of 10 μg/ml (personal observation). IL-6 functions as a second signal for IL-2 production by T cells (104).

In addition, monocytes express the IL-2*R*, and IL-2 has been suggested to regulate monocyte differentiation (146). CS might modulate this function through a block of IL-2 release by T cells.

e. MHC expression. Although the expression of MHC class II molecules was reported to be reduced by CS (124), and Whisler et al. (425) noted that human monocytes, pretreated for 18 h with CS (6 μg/ml or less), had a reduced expression of class II MHC antigens, due to an increase in PG synthesis, other studies have not confirmed these findings. Palay et al. (312), Lu et al. (240), and Granelli-Piperno et al. (120) did not find any direct effect of CS on the expression of MHC molecules induced by lymphokines. In agreement with this observation, CS does not block MHC class II antigen expression on thyrocytes or endothelial cells induced by IFN-γ (124, 321, 418). Moreover, a modulation of MHC expression has not been implicated as the mechanism by which CS would inhibit antigen presentation (240, 312).

However, CS exerts an indirect effect on the expression of MHC class II antigen by inhibiting lymphokine production by T cells. MHC class II expression on monocytes is not constant (372, 373) and is enhanced by IFN-γ (434) and IL-4 (57). In addition IFN-γ increases the expression of MHC class I gene products and induces the appearance of MHC class II gene products on many other cell types (340). MHC expression, particularly in response to lymphokines, is labile (194, 371), and continuous stimulation seems to be required for the expression of MHC class I and II products. Three relatively distinct pathways for altered systemic MHC expression in response to various external stimuli have been described: T cell release of IFN-γ, non-T cell release of IFN-α/β, and NK cell release of IFN-γ (173). The first and last mechanisms are inhibitable by CS (173). IFN-α/β, IL-4, and TNF may also be implicated in this process (57, 173). The reduction of expression of MHC class II antigens by endothelial cells and other cell types linked to the inhibition of lymphokine production by T cells might represent an important mode of action of CS in prolongation of allograft survival (124).

3. Antigen Processing and Presentation

In general, the doses of CS required to produce significant suppression of antigen presentation are higher than those required to inhibit lymphokine production. Antigen processing and presentation represent the "primum movens" of a specific T cell response. Whether the response will involve predominantly $CD4^+$ or $CD8^+$ T cells, it may depend on many variables, among which the antigen and the accessory cell are probably critical. The physiochemical properties of the antigen will determine whether it permeates all tissue microenvironments, whether it readily induces costimulators and whether it requires uptake and processing by accessory cells. The handling of antigen by accessory cells is a complex process involving several events: encounter, uptake, processing, binding to MHC molecules, reexposure on the cell surface, presentation to TCR, and production of soluble mediators, such as IL-1, IL-6, and other less well defined costimulatory signals. Concerning the accessory cell, the level of class I or class II MHC molecules, processed antigen, and in particular the costimulators expressed, will be essential in determining the interaction with the different subsets of T cells. In spite of a few controversial data, it is widely accepted to separate "exogenous antigens," taken up via endocytosis, from "endogenous antigens," newly synthesized within the cell (108). Antibody accelerates the rate of phagocytosis and antigen presentation because of the presence on accessory cells of Fc receptors which react with immune complexes. The rules may be slightly different in a transplanted tissue because the MHC antigens on the cell surface become themselves the most relevant foreign antigens.

In the case of exogenous proteins, accessory cells endocytose antigen molecules into acidic vesicles where proteins are denatured and fragmented by proteolytic enzymes into peptides of a size appropriate for interaction with MHC molecules (10 to 12 amino acids). Afterward, these complexes are exposed on the surface of the accessory cell accessible to TCR (201). The MHC system codes for glycoproteins slightly homologous to immunoglobulin (Ig) domains and probably developed into an elaborate system for presentation of foreign antigens to

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immunocompetent cells. The other important characteristic of the system is that only those linear molecular fragments, which can be presented properly in the context of the MHC, are recognized as foreign and can initiate an immune response (genetic responder or nonresponder). Antigen presentation for helper/inducer T cells ($CD4^+$), which, when activated, secrete growth and differentiation factors for other cells, requires the presence on the accessory cell of antigenic peptides associated with MHC class II gene product. Each MHC class II molecule consists of two highly polymorphic transmembrane glycoprotein chains of approximately 34 kDa (α) and 28 kDa (β) [three MHC class II genes are known in man (HLA-DR, HLA-DQ, HLA-DP) and two in mice (H2-IA, H2-IE); these loci code for α- and β-chains]. These molecules are permanently expressed only on a limited number of cells including macrophages, dendritic cells, Langherans' cells, B cells, and activated T cells and were originally defined on the basis of typing by mixed lymphocyte culture. However, they can be expressed on venous endothelial cells, keratinocytes, gut epidermal cells, thyrocytes, and other cells after appropriate stimulation with IFN-γ (8, 321, 324, 418). Functionally, the possibility to express MHC class II antigen on other cell types is relevant because it leads to localization and amplification of an ongoing immune response via MHC class II-restricted helper T cells. In vitro, MHC class II molecules are capable of stimulating proliferation of allogeneic helper T cells and are responsible for primary and secondary MLC reactions.

The other major subclass of T cells, suppressor/cytotoxic T cells ($CD8^+$), also requires the processing and presentation of antigenic fragments by the cell that is both the stimulus and the target of their lytic function, but in the context of the ubiquitously expressed MHC class I gene products (HLA-A, B, C in man and H-2K, D, L in mice; these closely linked loci code for different polymorphic transmembrane glycoproteins of 44 kDa—α-chains—which are always linked to another gene product the nonpolymorphic β_2-microglobulin of 11.5 kDa), which are the classic, serologically defined histocompatibility markers on which tissue typing was originally based. It is generally assumed that antigen association with class I structures is restricted to proteins which are newly synthesized within the cell (endogenous antigens).

Antigen-presenting cells are in highest concentration in lymphoid organs such as lymph nodes and spleen. These are the sites where lymphocyte traffic is more intense and where most contacts between antigen and reactive lymphocytes take place. However, in chronic inflammation, deposits of lymphoid and accessory cells can occur anywhere in the body.

The frequency of T cells responding to conventional antigens is usually low, because of the relative rarity of receptors with the particular combination of variable elements to match the antigen/MHC ligand. The study of TCR-ligand interaction is facilitated by the use of T cell lines or hybridomas which do not require accessory signals. In vitro the effect of CS on APCs has been evaluated using different assays such as the proliferative response of antigen-specific T cell lines, the release of IL-2 by T cell hybridomas, and the proliferative response of PBMC to antigens, mitogens, and autologous and allogeneic mixed lymphocyte reaction (MLR). Different antigens and accessory cells have been used in these studies (table 2).

In all these experiments, the carryover of minute amounts of CS represents the most critical aspect. With a few exception, it has been necessary to treat APCs with moderate to high amounts of CS (1 μg/ml or more) to observe a significant block in antigen processing and presentation; in contrast, T cell proliferative responses are inhibited by low doses of CS (0.05 μg/ml or less). The different sensitivity of T cells and macrophages to CS complicates the interpretation of the data because even a minor carryover of the drug may affect the results.

Some authors propose that antigen presentation is affected by CS (103, 247, 312, 425), whereas others show that antigen endocytosis, processing, and presentation are not directly or substantially modified by doses of CS that are used in most in vitro experimental protocols (240, 279). In these studies, a carryover of CS was carefully excluded. Lu et al. (240) explained all apparent effects of CS on antigen presentation and subsequent T cell proliferation by a residual amount of CS in the CS-treated accessory cells in the order of 0.01 μg/ml (2 to 5% of the input CS), which is enough to inhibit T cell proliferative responses. Only large doses of CS (9 μg/ml) directly effected antigen presentation, but at this dose a cytostatic effect was observed (103, 240). Muller et al. (279) used APCs belonging to the B lymphocyte lineage and found that thorough washing of the cells and incubation in fresh culture medium for 6 h was necessary to eliminate all CS traces. These investigators concluded that CS did not disrupt the processed antigen molecules, MHC molecules, IL-1, and any structure on the cell surface that was important for antigen presentation (240).

However, other studies suggest the possibility that CS, being lysosomotropic, might limit antigen catabolism and affect antigen processing and presentation (83). Moreover, mixing experiments in which CS-untreated antigen-pulsed APCs are cocultured with CS-treated monocytes and T cells tend to exclude the carryover of inhibitory amounts of CS (82, 408), and in these studies an effect of CS on antigen presentation was observed.

In addition, a few other points might be relevant to interpret these controversial results. The sensitivity to CS may depend on the type of antigen or of antigen processing. Esa et al. (82) showed that allogeneic MLR or the presentation of cytomegalovirus antigen were more sensitive to CS than antigen presentation of tetanus

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TABLE 2
Cyclosporine and antigen presentation

APC	Species	Antigen	Read-out	CS concentration (μg/ml)	Reference no.
Small monocytes	Man	Tetanus toxoid	T cell proliferation	1	83
Large monocytes		Tetanus toxoid	T cell proliferation	1	
Irradiated PBMC		Purified protein derivative	Proliferation of T cell line	0.5	246
			Lymphocyte proliferation	1	
Irradiated monocytes		Tetanus toxoid	Lymphocyte proliferation	1	82
		Diphtheria toxoid	Lymphocyte proliferation	1	
		Cytomegalovirus	Lymphocyte proliferation	1	
		Allogeneic MLR	Lymphocyte proliferation	1	
Monocytes		Tetanus toxoid	T cell proliferation	0.1	367
Irradiated monocytes		PHA	T cell proliferation	0.1	403a
		Con A	T cell proliferation	0.1	
		PWM	T cell proliferation	0.1	
		Sodium periodate	T cell proliferation	0.1	
		A23187	T cell proliferation	0.1	
		PMA	T cell proliferation	>0.1	
		Neuraminidase-galactose oxidase	T cell proliferation	0.1	
Monocytes*		Allogeneic MLR	T cell proliferation	>2.5	425
		Autologous MLR	T cell proliferation	1	
Monocytes		Neuraminidase-galactose oxidase	T cell proliferation	1	403
Langerhans' cells	Mouse	Con A	T cell proliferation	5	103
Peritoneal cells		*L. monocytogenes*	Proliferation of T cell line	9	240
Peritoneal cells		*E. coli* beta-galactosidase	IL-2 production by T cell hybridoma	0.5	246
B lymphoma line		Hen egg white lysozyme	IL-2 production by T cell hybridoma	>1	279
Irradiated spleen cells		Synthetic peptide			
Peritoneal macrophages		*L. monocytogenes*	IL-2 production by peritoneal T cell	>1	312
Irradiated spleen cells		Thyroglobulin	Proliferation of T cell line	0.1	408
		Purified protein derivative	IL-2 production by T cell line		
Irradiated spleen cells		*E. coli* beta-galactosidase	Proliferation of T cell line	0.5	247
Peritoneal macrophages			IL-2 production by T cell hybridoma		
Veiled cells	Rabbit	Con A	T cell proliferation	0.1	199

* Mitomycin treated.

toxoid. This may indicate that antigen processing and presentation are more critically affected by CS in the response to complex cell-associated MHC antigens or viral antigens than to soluble antigens. However, in this same report, the presentation of another soluble antigen (diphtheria toxoid) was sensitive to the effect of CS (1 μg/ml) (82) and in another study preexposure of monocytes to CS (0.1 μg/ml), during tetanus toxoid processing, led to marked inhibition of T cell proliferation (367).

In humans, CS appears to have differential effects on uptake of soluble antigens via Fc receptors (in the form of immunocomplexes—CS resistant) as opposed to via pinocytosis (CS sensitive) (246). These authors suggest that the drug probably interferes with the early steps of endocytosis-mediated antigen uptake and processing. However, direct experimental evidence that the endocytotic process is a possible target for CS has not been presented. In contrast, Palay et al. (312) suggest that CS interferes with a later stage in antigen processing, i.e., the complexing of processed antigen with MHC molecules. On the basis of some of these data, it would be possible to propose that the sensitivity to CS of antigens of different size and physical state (soluble, particulate, membrane bound, complexed to an antibody) might be variable. However, the reports of Lu et al. (240) and Muller et al. (279) tend to exclude any differential effect of the drug on particulate or soluble antigens.

CS might differentially inhibit antigen presentation mediated by various types of accessory cells interacting with different subsets of T cells. In both APCs and T cells, the requirement for stimulatory or accessory signals may be different. For example, macrophage cell surface MHC and antigen-presenting ability is enhanced by IFN-β (402), whereas B cell MHC class II gene expression is enhanced by IL-4 and inhibited by IFN-γ (270). Macrophages, various types of B lymphocytes, and dendritic cells have major differences in their capacities to process particular antigens and to present antigen to different clones of T cells (50, 402). Furthermore, differences in accessory cell requirements for Th1 and Th2 cells have been described and these are not limited to a given accessory cell lineage (i.e., macrophage or B cell) but rather to the state of activation of the accessory cells, particularly regarding the display of distinct costimulatory signals. Th1 clones do not require IL-1 for growth, but they require a costimulatory activity, not constitu-

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tively expressed and unrelated to the levels of class II MHC molecules or IL-1, to produce IL-2 (172, 416). The activation of Th2 cells is dependent on the expression of IL-1 by accessory cells (122, 213, 416). The same T cell clone may even be stimulated to different functions depending on the type of accessory cell-presenting antigen (9). Palay et al. (312) reported that CS inhibited macrophage-mediated antigen presentation to freshly prepared T cells which require accessory signals not needed by T lymphocyte lines (51, 78, 404). However, it is difficult to reconcile these data with the observation that CS does not affect directly accessory signals such as those delivered by soluble or membrane-bound IL-1 (82, 103, 367).

Other studies have shown the existence of functionally distinct human peripheral blood monocyte populations, i.e, small and large monocytes (83). Functionally, large monocytes present antigen less efficiently than small monocytes, they seem more resistant to the effect of CS (1 μg/ml), and antigen presentation by these cells is not sensitive to indomethacin (83, 150). On the other hand, antigen presentation by small monocytes is partially sensitive to indomethacin, and PGE might be involved. These authors, therefore, have suggested that CS may inhibit antigen presentation at more than one site and in different ways, because they were able to identify indomethacin-sensitive and -insensitive pathways in small and large monocytes (83, 150). A role for PGE_2 in the CS-mediated inhibition of antigen presentation by monocytes was proposed also by Schultze et al. (354) and Whisler et al. (425) but excluded by Palay et al. (312) and Snyder et al. (367). Dendritic cells and Langerhans' cells may represent other particular cases. Evidence provided by several groups (102, 103, 199, 408) indicates that preincubation with CS alters the antigen-presenting capacity of rodent dendritic cells and of isolated murine and human Langerhans' cells. Dendritic cells might be particularly important, because they seem to have a prominent role in rejection of vascularized organs.

In conclusion, a unitary interpretation of the data does not seem feasible because many aspects of the problem, such as the influence of different antigens, APCs, T cell subsets, mediators of inflammation, and other accessory molecules (IL-1, IL-6, LFA), should be better defined. Interindividual variations may explain some of these controversial results. In the case of a vascularized organ transplant, antigen processing and presentation probably follow the same general rules with some peculiarities linked to the location, size, variability, and nature of the antigens present in the tissues. In the experiments in vitro, a particular attention should be paid to avoid carryover of the drug, because most studies show that a dose of CS of 1 μg/ml or higher is required to influence antigen processing or presentation (table 2). As previously shown, these levels are probably exceeded in animal tissues and can be found also in some human tissue (74). However, because the serum levels of CS usually found in clinical practice are lower and lymphoproliferative responses which are independent of accessory cells are highly sensitive to CS, it looks unlikely that the primary mechanism of action of CS is through an inhibition of antigen processing, although in some cases (particularly high blood levels, selected antigens, or presenting cell) an inhibitory effect cannot be completely ruled out. In addition, studies claiming an effect of CS on antigen processing suggest different mechanisms of action so that no unifying hypothesis to explain the possible effect of CS in antigen presentation has been agreed upon.

These conclusions do not exclude, however, that in vivo CS, through its ability to block the secretion of several lymphokines and subsequently to inhibit MHC expression on other cells, may be instrumental in modifying and limiting ongoing antigen presentation in the organ and in the draining lymph nodes. In this case, notwithstanding the capacity to process and present antigen at the level of the cell, CS might have a considerable indirect effect in inhibiting in vivo the sensitization to alloantigens.

C. T Cells

1. T Cell Activation

a. Introductory remarks. Different T cell subsets are responsible for delayed type hypersensitivity, cytotoxicity, the regulation of many B and T cell functions, and the control of many other cell types. Cell activation and proliferation represent the cardinal events of the immune response. The early experiments of Borel et al. (30) showed that CS inhibited the proliferative response to mitogens such as Con A or PHA. Subsequent studies documented the role of CS in inhibiting cell activation and proliferation (80, 118, 202, 220, 235, 429), the pivotal role of ILs and of their receptors in mediating proliferation, and the ability of CS to block selectively early steps in lymphocyte activation and lymphokine gene expression (37, 216, 235).

In T cell proliferation two major levels of control are apparent. The first level is initiated by triggering of the TCR/CD3 complex, which leads to the expression of c-*myc*, lymphokines such as IFN-γ and IL-2, and IL-2*R* genes (activation). The second level starts with the binding of growth factors, IL-2 in particular, to their receptors. This second event is ultimately responsible for initiating an orderly and sequential program of gene activations and intracellular events which causes proliferation (amplification).

The action of CS on T cells is complex and has not yet been completely clarified. However, several important features of CS have been elucidated. First, CS is not lymphotoxic. Even after several days of culture with CS, lymphocytes are still capable of responding to proliferative stimuli when the drug is washed away. Second, CS at low concentrations (0.05 μg/ml) inhibits lymphokine

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production by selectively interfering with intracellular pathways necessary for gene expression. Third, CS at different concentrations may display disparate effects on various T cell subsets or clones, on different stages of activation in the same cell subset, and on different modes of activation. Fourth, CS inhibits in a dose-dependent fashion the lymphocyte proliferative response to mitogens or (allo)antigens with an apparent selectivity for T cells. Fifth, CS does not interfere with activation of Ts cells in the primary MLR. Sixth, CS strongly inhibits the proliferation of resting T cells but has limited effects on activated T cells (37). Seventh, the in vitro immune suppressive effect of CS can, at least in part, be explained by the functional disruption of ligand-receptor systems, mainly IL-2/IL-2*R*, which control the growth and the differentiation of (allo)antigen-reactive T cells. Eight, the actions of CS do not involve generalized alterations in membrane structures or interference with TCR/CD3 expression. Ninth, CS must be added early, in the first period of the culture, to obtain maximal inhibitory effect.

In mitogen-stimulated cultures, essential events, which are sufficient for DNA synthesis and morphological changes, occur during the first hours. Delay in the addition of CS by 1 to 2 h causes a significant reduction in the ability of CS to suppress lymphoproliferation, indicating that CS interferes with the expression of immediate and early genes (55). During this period, inhibition is completely reversed when the drug is washed away. On the other hand, CS only partially inhibits IL-2-dependent growth of activated T cells, indicating that it has much less effect on the expression of late genes (37, 221, 302). In contrast, the primary MLR is less dependent upon the time of addition of CS. Maximal suppression of the lymphocyte response to alloantigens is observed when CS is added within the first 96 h of culture.

b. Signaling pathway. At the first level of control, different ligand-receptor interactions at the outer side of the cell membrane are involved in T cell activation. The stimulation of the TCR/CD3 complex by anti-CD3 monoclonal antibody (MAb), alloantigens, or protein antigen fragments in the context of MHC class I or class II molecules represents the most physiological signal. Secondary accessory signals are not limited to IL-1 and are normally provided by APCs. For example the primary activation signal to $CD4^+$ T cells is given via the TCR/CD3 complex by antigenic peptide fragments together with MHC class II molecules and secondary signals via interaction between CD2 on the surface of T cells and LFA-3 in the membrane of an accessory cell (132). In this case, the two signals are given via the APCs and thus almost simultaneously. These initial events may be even more complex because they can be mediated by other ligand/receptor systems.

Inside the cell, rapid changes in cation fluxes (expecially changes in free cytosolic calcium $[Ca^{2+}]_i$), phosphatidylinositol hydrolysis, membrane translocation of protein kinase C, increased cyclic adenosine monophosphate concentrations, and enhancement of nucleoside uptake occur (88, 106, 176, 226, 227, 385). It is believed that also inside the cell at least two signal pathways are activated to deliver the proper stimuli for coordinate gene expression. These pathways are initiated, respectively, by an increase in $[Ca^{2+}]_i$ flux and by activation of protein kinase C. Some stimuli such as calcium ionophores and phorbol esters can bypass the ligand-receptor interactions on the cell membrane and can directly increase $[Ca^{2+}]_i$ flux and activate protein kinase C, respectively (186). PHA activates both pathways, whereas certain anti-CD2 MAbs (161) or the combination of IL-4 and phorbol esters (165) can induce T cell activation independently of $[Ca^{2+}]_i$ increase in the cytoplasm. The increase in $[Ca^{2+}]_i$ activates or facilitates the activities of several cell proteins and enzymes. Much work suggests that CS almost completely inhibits lymphocyte activation by agents that induce $[Ca^{2+}]_i$ flux (184) but not by agents, such as phorbol esters, activating directly protein kinase C (187). The role of CS in inhibiting the early increase in $[Ca^{2+}]_i$ flux has been debated. Gelfand et al. (106) reported that 30 min of preincubation with CS blocked $[Ca^{2+}]_i$ flux, an obligatory event for the PHA-induced expression of IL-2 gene. Similarly, Isakov et al. (169) showed indirect evidence suggesting that CS would affect the initial increase in $[Ca^{2+}]_i$. In contrast, Redelman (329) showed that 30 min of preincubation with CS concentrations sufficient to inhibit 90 to 100% proliferation has no effect on $[Ca^{2+}]_i$ increase and on changes in cell volume that occur in human PBMC-stimulated by PHA. Moreover, in murine T cells, 10 min of preincubation with CS does not prevent the increase in $[Ca^{2+}]_i$ induced by Con A (260), and the increase in $[Ca^{2+}]_i$, secondary to anti-CD3 MAb binding to Jurkat cells, is not inhibited by CS (433). In addition, CS prevents lymphocyte activation by ionophores which directly cause an increase in $[Ca^{2+}]_i$ (186). Other studies have suggested that CS blocks $[Ca^{2+}]_i$ influx-dependent Na^+/H^+ exchange (342). In conclusion, it is now accepted that CS does not directly affect $[Ca^{2+}]_i$ flux but interferes with one or more subsequent steps along the pathway that begins with an increase in $[Ca^{2+}]_i$, and, later on, leads, in an organized fashion, to the synthesis and secretion of lymphokines. The signaling pathway that apparently links protein kinase C with lymphokine secretion does not appear to be influenced by CS because potentiation of mitogen responses by PMA is not inhibited by CS (269).

c. Lymphokine secretion. These early events are followed by an activation cascade which leads to lymphokine production, to their secretion into the extracellular medium, to the induction of cell surface receptors for the growth factors, and to subsequent proliferation (42, 222). During this process T cells undergo morphological

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changes, divide, and differentiate as specific genes are sequentially activated during several days. More than 70 molecules are specifically regulated during this process (55). CS inhibits the synthesis of mRNA for lymphokines and other proteins required for cell proliferation (202). Inhibition of transcription of lymphokine genes such as IL-2 (37, 80, 119, 202), IFN-γ (84, 118, 145, 334), IL-3 (145), probably GM-CSF (16, 315, 359), IL-4 (120, 160, 217, 361), IL-5 (earlier called TRF or BCGF-II) (119), TNF-α, TNF-β (84), lymphocyte-derived chemotactic factor (182, 216), and B cell differentiation and cytotoxic differentiation factor (37, 119, 178) is the most important consequence of CS activity. Information concerning the effect of CS on the production of IL-6 by T and B cells (159), neuroleukin (131), IL-7 (285), or P40 glycoprotein (404), which, except IL-7, are secreted by T cells, is not yet available. Now, several factors that promote the activation and the growth of T cells are known such as IL-2 (273), IL-4 (116), IL-6 (237), GM-CSF (211, 436), IL-7 (275), and P40 glycoprotein (404). The best defined T cell growth factors are IL-2 and IL-4. These lymphokines by interacting with their specific receptors provide a second level of control that generates additional intracellular signals capable of activating other genes essential for cell growth and differentiation (297, 314, 339). IL-2 is a lymphokine secreted in an early period after T cell activation, which in vitro facilitates or permits clonal expansion of T cells, including both $CD4^+$ and $CD8^+$ subsets. In addition, IL-2 stimulates T cells to up-regulate the IL-2*R* (Tac) (245) and to produce other lymphokines such as IFN-γ (164, 166). It stimulates B cells and monocytes (283, 327), but the most thoroughly studied activity is its ability to support the development of CTL (81). IL-4 is a second lymphokine which can drive proliferation of cloned CTL (116), of cloned Th cells (89, 116), and of activated $CD4^+$ and $CD8^+$ populations (165, 356a). IL-4, in contrast to IL-2, has no detectable lymphokine-activated killer (LAK)-inducing activity (427, 428). It is possible that IL-2 and IL-4 exert their effects on different cell subsets and, perhaps, at different stages of cellular activation (427, 428).

Most studies have evaluated the role of CS on IL-2 synthesis and secretion. The expression of the IL-2 gene is strictly dependent on signals from the TCR/CD3 complex. Ionophores and $[Ca^{2+}]_i$ increase lead to IL-2 production rather than IL-2*R* expression (106, 265, 267). Apart from an initial study (221), all subsequent studies (37, 70, 151, 325) based on various biological assays have demonstrated that CS inhibits the production of lymphokines and, in particular, IL-2. However, in some studies, CS (1 μg/ml) does not completely abolish IL-2 secretion in activated T cell culture supernatants, because a certain amount, about 20%, of biological and immunological intact IL-2 is still detectable (325).

This observation suggests that additional events important for growth are also blocked by CS. The time kinetics of IL-2 production remain unaltered (325). The mechanism of action of CS in the case of IL-2, and probably of other cytokines, involves a rapid blockade of the transcription of the relevant genes. Specific inhibition of transcription of several genes in the absence of a generalized blockade of cellular transcription is one of the characteristics of the specificity of CS (118, 119, 202). However, in several systems, inhibition of IL-2 production cannot be the sole mode of action of the drug because supplementation of cultures with exogenous IL-2 does not restore proliferation (106).

d. IL-2R expression. The expression of the IL-2*R* gene is an important indicator of the activation state of the cells and is essential for commitment to cell division and immunologic function (358). The effect of CS on the expression of the IL-2*R* still represents a controversial issue. The mechanism by which mitogens or antigen induce proliferation of T cells can be dissected into two major events: a rapid production of IL-2 and a delayed acquisition of responsiveness to IL-2 (IL-2*R* Tac expression) (204). Similar results were obtained by Granelli-Piperno et al. (118) who showed that the expression of IL-2 and IFN-γ precedes the expression of IL-2*R*. Naive PBMC do not express detectable levels of IL-2*R* (Tac) on their surfaces, but its expression can be detected within 24 h after mitogen stimulation (235). IL-2 and IL-2*R* gene expression are independently regulated in T cells (66, 175, 181, 251), and IL-2*R* (Tac) expression and IL-2 induced T cell proliferation may occur in the absence of changes in $[Ca^{2+}]_i$ (264, 265). CS has no direct effect at this second level of control, i.e., after interaction of IL-2 with its receptor.

Proliferation in response to IL-2 requires the formation of a trimolecular interaction among IL-2 and a high affinity IL-2*R* complex which includes at least two distinct polypeptide chains: a 55-kDa subunit (p55; α-chain) recognized on the cell surface by the anti-Tac MAb (Tac) and a 75-kDa subunit protein (p75; β-chain) (338, 339, 364, 386). Individually expressed α- and β-chains bind IL-2, with low and intermediate affinity (398, 415). The α-chain alone is nonfunctional, whereas the β-chain is pivotal in the transduction of the IL-2 and in mediating its internalization. Cells expressing the β-chain alone can respond to IL-2 (397), and the β-chain is constitutively expressed without the α-chain on the surface of several cells including some resting T cells and peripheral blood NK cells (291, 358, 397). When both chains are expressed together, high affinity receptors are detectable. They make up only a minority of the total IL-2*R* (339), and they are formed only after the expression of the α-chain (Tac) that is tightly regulated by antigen or mitogen stimulation. Different T cell subsets differ in their requirements for α-chain expression, and the α-chain is detected primarily on activated T cells (266). Two signals are generally involved in the expression of the α-chain (Tac) on T cells (396, 421). One signal is provided by

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perturbation of the TCR, whereas the second may be given by phorbol esters (PMA). However, the α-chain is also expressed after cell activation with only one stimulus such as physiologic activators of protein kinase C, PMA (66), TNF (228), IL-1 (323), and IL-5 (238).

The capacity of CS to affect the expression or function of the IL-2*R* remains controversial. The heterogeneity of the results suggest that the effects of CS on IL-2*R* (α- and β-chains) are complex as are the regulatory mechanisms controlling their expression. A better definition of the stimuli involved in the expression of the β-chain (p75) might be useful to solve some of these controversies.

Various direct or indirect methods have been used to assess the effect of CS on the expression of the IL-2*R*. Early investigations relied on the acquisition of IL-2 responsiveness evaluated as rescue of proliferation or capacity of the cells to absorb exogenous IL-2. These tests assess mainly the appearance of functional receptors. Later, the expression of surface IL-2*R* (Tac) with different anti-CD25 MAbs and the expression of IL-2*R* mRNA (α-chain) were studied. Number of positive cells and intensity of staining with MAb were assessed. Anti-CD25 MAbs recognize both the biologically active high affinity form (α- and β-chains) (233, 423) and the less important low affinity form (α-chain alone). More recently, the expression of high affinity IL-2*R* with radiolabeled IL-2 has been analyzed. Information on the expression of the β-chain is mainly indirect and obtained through the use of the latter method.

Some authors (37, 153, 267, 301) have reported that CS-mediated impairment of T cell proliferation in response to (allo)antigen or mitogen stimulation was fully overcome by the addition of exogenous IL-2 and suggested that the effect of CS on IL-2*R* expression was minimal. On the contrary, other studies (70, 154, 185, 221, 235, 309, 310, 407) showed that reconstitution of the culture with doses of IL-2 that are in excess of the levels measured did not reverse CS-mediated inhibition of proliferation. Rescue of proliferation is more pronounced at low CS concentrations (235). Previous studies (37, 154, 221, 235, 302) excluded that CS interferes directly with IL-2*R* function in that CS fails to suppress IL-2-induced proliferation in long-term cultures of activated T cells.

In human systems, three groups (267, 346, 407) found normal IL-2*R* (Tac) expression in lectin- and anti-CD3-stimulated PBMC. In another study, CS did not affect the number of IL-2*R* (Tac)-positive cells after lectin stimulation but reduced the intensity of expression on cells of all sizes (369). However, most studies in human and murine systems show a partial or marked reduction in IL-2*R* (Tac) expression in mitogen- or anti-CD3-stimulated T cells (3, 25, 41, 105, 191, 231, 235, 325, 326, 329, 330, 333). The reduction in the relative number of IL-2*R* (Tac)-positive cells affects the $CD4^+$ and the $CD8^+$ subgroups equally (325). As IL-2 up-regulates IL-2*R* (Tac) expression on stimulated cells, in the presence or absence of CS (65, 365), it is possible that CS-mediated inhibition of IL-2*R* (Tac), at least in part, reflects inhibition of IL-2 production (326, 333). Another possible explanation for the disparate findings is that IL-2*R* (Tac) expression varies with time, but also in this case contradictory results have been found. Lillehoj et al. (235) in a murine system found near normal levels of IL-2*R* (Tac) 24 h after mitogen stimulation and much lower levels 72 h after stimulation. In contrast, Calder et al. (41) showed that, already 24 h after stimulation, the number of IL-2*R* (Tac)-positive cells is diminished in a dose-dependent fashion, and Redelman (329) reported that the inhibition of IL-2*R* (Tac) expression is maximal in the first day of culture and that PBMC cultured with PHA in the presence of CS recover expression of activation antigens on day 3. As expected, CS does not inhibit IL-2*R* (Tac) expression in an already activated human leukemic T cell line (Jurkat) (202); however, in vivo, CS inhibits IL-2*R* expression and proliferation of lymph node T cells stimulated by Con A (24).

In contrast to the findings of the expression of the Tac antigen on the cell surface, which is decreased in most studies, CS does not seem to inhibit IL-2*R* α-chain expression at the transcriptional level. In PBMC stimulated with lectins and PMA, or anti-CD3 MAb, CS has only a marginal effect on the mRNA levels of the α-chain (55 kDa) (117, 118, 120, 330). However, a strict correlation between cytoplasmic levels of mRNA and surface expression might be misleading. In the same study, expression of the Tac antigen on the surface was found to be depressed in spite of slightly reduced mRNA levels (120). These data suggest that CS might interfere with some posttranscriptional event. Cyclophilin, a CS-binding protein involved in protein folding (96, 380), might be a possible candidate for this effect. No information is available about the effect of CS on the expression of the IL-2*R* β-chain mRNA. An alternative explanation might be an inhibition of the IL-2*R* (β-chain) by CS. Also in this case, the consequence would be a functional block of the IL-2*R*. It is important to note that the expression of the IL-2 gene is inhibited by CS in contrast to the expression of the IL-2*R* (α-chain) gene (330). This represents an additional indication that CS is selective in its action and affects in a different way the expression of different genes.

The effect of CS on the expression of high affinity IL-2*R* on the cell surface is controversial. The interpretation of the data becomes even more complex when one considers that IL-2 up-regulates the expression of the Tac antigen (α-chain) but may limit the expression of high affinity receptors in lectin-stimulated T cells (365). This indicates that expression of high and low affinity IL-2*R* is at least partly regulated via different mechanisms. With radiolabeled IL-2, Povlsen et al. (325), who stimulated human PBMC with purified protein derivative and

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PHA and in an MLR, clearly demonstrated that the pool of high affinity receptors is reduced in response to CS even if relatively high levels (1 μg/ml) must be reached to obtain a complete inhibition. Maximum inhibition was obtained when antigen and CS were added to culture medium simultaneously. Although the number of the high affinity IL-2*R* is reduced, the turnover of the remaining high affinity IL-2*R* is unaffected. Binding kinetics, affinity, uptake, and degradation of IL-2 were qualitatively unaffected by high doses of CS. This point supports the concept that CS has no effect on T cells once activated (153, 221, 301). No correlation was found between the expression of low and high affinity IL-2*R* (325), because anti-CD25 MAbs were unable to detect variations in the expression of high affinity IL-2*R*. CS mediates immunosuppression by affecting early events during T cell activation, and variations in high affinity IL-2 receptor expression and IL-2 production are secondary to this effect (325). Similar results have been reported by Reed et al. (330), who demonstrated that CS (0.5 to1.0 μg/ml) at concentrations that inhibit PHA-induced proliferation by 80 to 90% diminished by about 50% the expression of both high and low affinity IL-2*R* on human PBMC. On the other hand, Bloemena et al. (25) showed that, although CS (1 μg/ml) causes a decrease in Tac antigen expression in 48- and 72-h PHA-stimulated human PBMC, the expression of biologically active high affinity IL-2*R* is not affected. Similar data were reported by Ryffel et al. (345) who stimulated nylon wool-enriched populations of human T cells with anti-CD3 MAb and monitored the emergence of IL-2*R* in the presence or absence of CS. In the presence of CS they showed no reduction in anti-Tac-reactive cells. However, with the use of radiolabeled IL-2, they found a reduction in the number of low affinity binding sites and in most experiments no change in high affinity binding.

On the other hand, these same authors (25, 345) and several other ones (106, 184, 235, 326, 330, 407) clearly showed that CS-induced inhibition of lectin-stimulated PBMC is not reversed by the addition of doses of IL-2 that are in excess of the levels measured in optimally stimulated cultures. Apparently, expression of high affinity IL-2*R*, indicator of the activated state of the T cells, is in itself not sufficient for proliferation of the T cell, even when IL-2 is supplied exogenously. These data suggest that CS blocks the induction of other signals necessary for T cells to become capable of proliferating in response to IL-2. Transcription of other genes sensitive to the action of CS may be involved (118, 203). In addition, secretion of soluble factors other than IL-2, synthesis of regulatory proteins that associate with IL-2*R* in the membrane of T cells, expression of protooncogenes, and activation of intracellular protein kinases are among the numerous possibilities to explain these data (330).

In conclusion the analysis of these results suggests that CS influences the expression on the cell surface of low and high affinity IL-2*R*. Further studies are required to clarify the role of CS on the expression of the IL-2*R* (β-chain).

e. Other markers of T cell activation. CS exerts differential effects on the expression of other activation antigens. The transferrin receptor, the E-rosette receptor (CD2), the activation antigens defined by the OKT10 MAb, and the HLA-DR antigens are reduced by CS (15, 235, 267, 329). Marder and Schmidtke (249) found an inhibition of expression of CD4 and CD8 antigens on CS-treated Con A-activated T cells, whereas Leapman et al. (225) and Hess et al. (149) did not observe any effect on the same antigens in CS-treated alloreactive T cells. The expression of the 4F2 antigen which increases on activated cells at a very early stage (4 h) remains unchanged after CS treatment (15). The inhibition of the transferrin receptor gene may be due to an indirect phenomenon because the expression of this receptor appears to be a consequence of IL-2 interacting with its receptor (55). CS mainly influences expression of activation antigens on Th cells, whereas $CD8^+$ CTL are not influenced (15).

Newman et al. (287) described the appearance of three early activation antigens on the T cell surface. These markers appear before IL-2*R* (Tac). CS affects each one differently; it blocks the synthesis of Ea1, partially affects the expression of Ea2, but does not influence that of Ea3 (287).

In addition, CS interferes with other early events of T cell activation, including the expression of protooncogene (202, 331, 332) and of early intracellular enzymes, such as ornithine decarboxylase (93).

f. T cell locomotion. Morphological polarization and locomotion of lymphocytes from the blood into tissue is an important step in immune inflammation. The activation of locomotor capacity of human blood lymphocytes by anti-CD3 MAb, PHA, and *Staphylococcus aureus* Cowan strain (SAC) is blocked by the presence of CS (0.01 μg/ml) (162). On the other hand, locomotion induced by PMA is CS resistant, as well as locomotion of lymphocytes that already possess this capacity (about 40% of resting human blood lymphocytes) (430). Locomotor capacity is acquired by 80 to 90% of PHA-activated lymphocytes, it occurs in the G_1 phase of growth, during the first 24 to 48 h of culture and requires protein synthesis but not DNA synthesis. Neither IL-1 nor IL-2 reverses the inhibitory effect of CS on anti-CD3-activated locomotion in culture (430). These data suggest that part of the action of CS might be linked to inhibition of lymphocyte recruitment into antigen-mediated inflammatory lesions.

2. *Mitogens, Antigens, and MLR*

a. Introductory remarks. CS at low concentrations inhibits the activation and the proliferative response of T cells of different animal species and humans. The dose

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of CS that inhibits 50% of the proliferative response to mitogens varies from species to species and may reflect a differential sensitivity among species (27). Most T cells prone to activation are suppressed by CS, regardless of phenotypical subtype, although different T cell subsets have different sensitivities. In addition, the mitogen used greatly affects sensitivity to CS (186).

Many stimuli, including lectins, antigens, anti-TCR/CD3 MAb, anti-CD2 or CD28 MAb, PMA, and ionophores, and the autologous and allogeneic MLR have been used to analyze the effects of CS on T cell function and proliferation. Some of these stimuli can directly drive purified T cells to proliferate, but most require the presence of APCs or a combination of multiple stimuli. The most studied stimuli are those inducing activation via the TCR/CD3 structure in the presence of accessory signals because they reproduce physiological responses. However, all these stimuli are useful to discriminate between different pathways of T cell activation.

Large differences of sensitivity to CS are observed depending on the nature of the stimulating signal and the differentiation state of the responding T cell (70, 186). The primary MLR response is the most sensitive to low CS doses, whereas secondary MLR responses and mitogen responses are more resistant. PHA response of human lymphocytes is the least affected (152, 154), and PMA responses are CS resistant (26). A strict correlation between the CS sensitivity of the response to mitogens (anti-CD3 MAb > Con A > PHA) and their dependence on accessory cells has been proposed (185); however, CS exerts a strong inhibitory effect on several monocyte-independent proliferative responses in which the requirement for accessory cells is substituted by PMA or by immobilizing anti-CD3 MAb (25, 26). As already mentioned, CS acts within the first hours after its addition to mitogen-stimulated T cells (184, 429). Inter- and intraindividual differences in CS sensitivity of PBMC activated by mitogens or antigens or in MLR have been reported (325, 349, 362, 444).

b. Anti-TCR/CD3 MAb. T cells can be activated by MAbs directed to the TCR/CD3 complex. Soluble anti-CD3 MAbs require accessory cells to induce proliferation, whereas stimulation of purified T cells with immobilized anti-CD3 MAb is sufficient to induce full proliferation (107). T cell proliferation induced by soluble or particulate anti-CD3 MAb is extremely sensitive to CS, with a 50% inhibitory concentration of about 0.025 μg/ml (175, 185, 429), although differences in sensitivity to CS among different anti-CD3 MAbs have been described (353). Complete inhibition of anti-CD3-activated T cells is obtained with CS concentrations of 0.1 μg/ml, and the addition of recombinant IL-2 increases the proliferative response and partially reverses the inhibitory effect of CS mainly at low CS doses (0.003 μg/ml) (132).

Only one MAb directed to the constant part of the TCR-α/β has been evaluated (353). This MAb induces mainly the stimulation of $CD8^+$ T cells and does not require cross-linking by macrophage Fc receptors, and its stimulatory activity is less sensitive to CS than that of anti-CD3 MAb (353).

In T cells stimulated by anti-CD3 or anti-CD3 plus PMA, IL-2 and IL-4 mRNA expression is completely abolished by CS (175, 217). Proliferation induced by anti-CD3 MAb in the presence of CS resistant stimuli such as CD28 or PMA results in less sensitivity to the drug (175, 217), but IL-2 gene expression is still abolished (117). Evidence has been provided of IL-2- and IL-4-independent proliferation in human T cells after activation via anti-CD3 MAb and PMA. This proliferative response is CS resistant (217).

T cells capable of suppressing in vitro antibody responses can be induced by anti-CD3 MAb (63). These Ts cells are radioresistant and CS sensitive (63).

c. Anti-CD2 MAb. A second pathway of T cell activation independent of the TCR/CD3 complex or the participation of IL-1 or accessory cells has been described. This pathway acts through the CD2 antigen. Binding of the CD2 ligand termed LFA-3 (356) or of MAb to the CD2 sheep red blood cell E rosette receptor results in activation of T cells and large granular lymphocytes (176, 261). Although some anti-CD2 MAb lead to T cell activation without a detectable increase in $[Ca^{2+}]_i$ levels (161), a combination of two anti-CD2 MAbs usually induces cytoplasmic Ca^{2+} increases (4). Probably, the CD2 pathway, presumably through ligands such as LFA-3, can activate T cells to proliferate in an antigen-nonspecific manner (176, 261, 356). CS inhibits in a dose-dependent fashion T cell proliferation and IL-2 production in both $CD4^+$ and $CD8^+$ T cells after activation via CD3 plus CD2 (132). The addition of recombinant IL-2 to the cultures abolishes the suppression to some extent (132). Under these culture conditions, the primary activation signal must be delivered through CD3 (132).

d. Antigens. Similar observations have been obtained with antigen-stimulated human and murine T cells (235). Antigens such as tetanus toxoid, purified protein derivatives, streptokinase, *Cryptococcus neoformans,* and ovalbumin have been used (33, 235, 263, 325, 388, 407). T cell responses are completely inhibited by CS (range 0.03 to 1.00 μg/ml), and the concentrations of CS that produce optimal inhibition of T cell proliferation are also effective at inhibiting IL-2 production, IL-2 responsiveness, and the expression of IL-2*R* (Tac) and transferrin receptor (235, 263).

e. Horse anti-human lymphocyte serum (ALS). In humans, ALS-induced proliferation of PBMC is inhibited by CS (26, 407). ALS is able to induce proliferation of T cells in the absence of APCs. IL-2 production induced by ALS is not inhibited significantly by CS (1 μg/ml), and addition of exogenous IL-2 does not reconstitute CS-induced inhibition.

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f. Con A and other CS-sensitive T cell mitogens. CS (0.05 to 0.5 μg/ml) completely prevents Con A-stimulated human, murine, and guinea pig T cells from proliferating and producing IL-2 (70, 235, 249, 309, 374, 407).

In one study, it was shown that CS suppressed the proliferative response and the generation of Ts cells induced by Con A (309), whereas other groups demonstrated that CS spared the activation of Con A-induced Ts cells (130, 223, 224). These controversial results may be linked to the different systems used to measure suppression (130).

Proliferation of PBMC induced by calcium ionophores (77, 379), dextran-sulfate (311), or pokeweed mitogen (PWM) (113, 224, 303) is sensitive to CS inhibition.

g. PHA. The plant mitogenic lectin, PHA, is a T cell mitogen that is dependent on accessory cells (431) and induces T cells to proliferate via multiple activation pathways which can be pharmacologically differentiated and have distinct lymphokine requirements (318). CS (1 μg/ml) inhibits only by 80% the proliferative response of enriched human T cell populations stimulated with PHA and does not completely inhibit IL-2 production (106, 113, 407). A residual CS-resistant proliferative response remains unaffected (152, 154). In the mouse, CS (0.1 μg/ml) reduces by less than 50% T cell activation induced by PHA. This CS-resistant proliferative component of the PHA response is carried out by a subset of T cells and is both IL-2 and IL-4 independent, and the lymphokine involved in this T cell activation pathway remains to be identified (318). Understanding the mechanism of CS resistance may clarify certain aspects of IL-2-independent T cell activation in vivo and help to define experimental and clinical situations in which CS treatment fails to suppress specific immune responses.

In the continuous presence of CS, the expression of the IL-2*R* (Tac) in PHA-stimulated T cells is inhibited by approximately 50%, and the addition of exogenous IL-2, in doses that are in excess of the levels measured in optimally stimulated cultures, only partially reverses the drug effect (25, 106, 235, 330, 407). The failure of IL-2 to restore proliferative responses of T cells suggests an additional site of action of the drug (106).

When the cells are exposed to CS (1 μg/ml) for 30 min, washed, and subsequently incubated with PHA, other phenomena become apparent. In these conditions, the expression of the IL-2*R* (Tac) is not affected, and exogenous IL-2 restores the proliferative response, implying the expression of functional IL-2*R* (106).

CS (1 μg/ml) virtually abolishes PHA-induced colony formation, and the addition of IL-2 does not restore clonal growth to normal (99, 114, 432). These data suggest that in this system the dominant activity of the drug results from a block in the expression of the IL-2*R* (Tac).

h. Phorbol esters [PMA, phorbol 12,13-dibutyrate (PDB)]. In primary cultures of unfractionated human PBMC, PMA is a T cell mitogen with a potency approximately one-third that of PHA (394). Lymphocyte populations extensively depleted of accessory cells still show some response to PMA, but at least 80% of the response is dependent on accessory cells (60, 187). In addition, PMA can act as a comitogen for T cells; activation in the presence of PMA is additive with that induced by PHA and can substitute for accessory cells (60, 187). The proliferation and IL-2 production induced by PMA is partially resistant to CS because it can be inhibited only about 50% by CS (1 μg/ml) (175, 187, 407). PMA leads to T cell activation by direct binding and activation of protein kinase C. This activation pathway does not result in an increase in intracellular $[Ca^{2+}]_i$ and is sufficient to induce expression of the α-chain of the IL-2*R* (66, 118).

The combination of phorbol esters (PMA or PDB) and the calcium ionophore, ionomycin, can induce proliferation in enriched T cell preparations which is almost completely inhibited (80%) by CS (50% inhibitory concentration 0.3 to 0.5 μg/ml) (204). In these conditions, IL-2*R* (α-chain) mRNA is detectable, but the proliferative response is not restored by providing exogenous IL-2, suggesting a defect in the expression of the IL-2*R* (β-chain) or another defect along this signaling pathway (106). T cells incubated with PDB and ionomycin for 30 min do not proliferate but become competent to proliferation induced by exogenously added IL-2 or PDB (204). CS (1 μg/ml) added in the initial incubation period abrogates the induction of T cell competence and prevents the expression of the IL-2*R* (α-chain) gene. CS does not affect the ability of already competent cells to proliferate to exogenously added IL-2 or PDB (204). Also,these data confirm that the effects of CS are primarily limited to the early phase of T cell activation.

i. Anti-CD28 MAb plus PMA. CD28 is another cell surface antigen expressed on most mature T cells, and in particular on 95% of $CD4^+$ and on 50% of $CD8^+$ T cells (58). The $CD8^+$, $CD28^+$ T cell subset does not contain Ts cells (58). The combination of CD28 stimulation and PMA results in T cell proliferation which is independent of both accessory cells and activation of the TCR/CD3 complex (134). T cell proliferation induced by CD28 appears to involve a T cell subset distinct from that induced by the TCR/CD3 (134). The CD28 signal synergizes early in the cell cycle with PMA, resulting in IL-2 gene expression. The pathway activated by CD28 plus PMA is resistant to CS (3 μg/ml) so that proliferation is not modified by the drug (175). IL-2 gene expression may be partially inhibited (117). A potential role for the CD28 molecule in vivo may be to augment IL-2 production after stimulation of the TCR/CD3 complex and, therefore, to amplify an antigen-specific immune response.

j. MLR. Among the various ways to stimulate T cell proliferation the allogeneic MLR has more similarities with the conditions encountered in an allograft. MLR

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can be seen as an in vitro allograft response that depends on MHC recognition and results in the generation of CTL capable of specifically recognizing and killing the target cells (CML). The CML assesses the destructive pathway following activation. As in an organ transplant, T cells are directly activated by major and minor histocompatibility antigens present on foreign cells. The frequency of T cells responding to allogeneic forms of MHC molecules is high (97), and the requirements for stimulating MLR differ from those of mitogen-stimulated cultures (95, 242). On mitogenic (118, 127, 132) or antigenic stimulation (268, 370), both $CD4^+$ and $CD8^+$ T cells proliferate and secrete IL-2. After allogeneic activation, the majority of IL-2-producing cells are of the $CD4^+$ phenotype, suggesting that IL-2 production by $CD8^+$ T cells is stimulus restricted and not inducible in an MLR (262).

The induction of CTL can be viewed as the sum of sequential steps: (1) presentation of peptides in the context of MHC class I and II molecules by APCs; (2) ligand-receptor interactions through accessory molecules such as IL-1, or LFA-3; (3) activation of class II-restricted MHC-reactive $CD4^+$ Th cells that release lymphokines, such as IL-2 and IFN-γ, and express receptors for growth factors; (4) proliferation of $CD4^+$ Th cells; (5) induction of competence in resting class I-restricted $CD8^+$ CTL precursors with subsequent expression of IL-2*R* but not IL-2 secretion (243, 412); (6) binding of paracrine-secreted IL-2 to the IL-2*R* with subsequent progression and clonal expansion of competent CTL; and (7) activation of radioresistant Ts cells and modulation of the response (54).

Lymphokines have a critical function. IL-2 induces both long-term proliferation of CTL (35, 36, 110) and differentiation of CTL precursors to the cytolytic state (81). IFN-γ is a maturation factor, and IL-2 and IFN-γ cooperate in the generation of CTL (381). IL-4 acts as a proliferative and differentiative signal for CTL generation (116, 319, 428), and IL-5 affects some aspects of CTL differentiation (382).

In murine, porcine, and human primary MLR, CS inhibits several steps in this process such as lymphokine secretion by Th cells, induction of competence in CTL, and in an indirect way, IL-1 secretion by APCs. Other events are spared such as progression of CTL and activation of Ts cells. The final result is a block of proliferation and of generation of alloreactive CTL (37, 123, 148, 162, 286, 414, 426). In humans, large interindividual variation in the sensitivity of CTL precursors to CS have been reported (177, 349), whereas interstrain differences were not observed in mice (349). In man, the generation of CTL, measured by a CML assay, is more sensitive to inhibition than cell proliferation, measured by [^{3}H]thymidine incorporation (151, 349). These differences are not so evident in mice (349). The generation of specific allocytotoxicity, i.e., the one directed against stimulator cells, is more influenced by CS than the generation of a specific allocytotoxicity versus third-party target cells (11, 219).

Proliferation of both $CD4^+$ and $CD8^+$ T cells is affected when CS is added during the first 72 to 96 h of culture (149, 152). In the presence of CS (0.01 to 0.05 μg/ml), Th cells do not secrete IL-2 and CTL are not generated (37, 148). Therefore, one mode of inhibition by CS is indirect with respect to the $CD8^+$ CTL themselves, being directed at inhibiting the secretion of lymphokines by $CD4^+$ Th cells. In addition, CS prevents the release of IL-1 from macrophages by an effect on Th cells, presumably by inhibiting the release of T cell lymphokines which activate macrophages to release IL-1 (138).

At higher concentrations, CS has a direct inhibitory influence on the activation of CTL precursors. In murine and human systems, the CS-mediated suppression of CTL generation is reversed by IL-2 only at low levels of CS (0.05 to 0.25 μg/ml depending on the study) (11, 37, 70, 148). At higher levels of CS (0.5 μg/ml), irreversible inhibition is seen even when an excess of exogenous IL-2 is added (34, 37, 148). This suggests an interference of CS with the expression of functional IL-2*R* or with other mechanisms essential for the acquisition of competence by CTL. CS must be present within the first 18 to 24 h to prevent the sensitization and activation of CTL precursors (148). After this period, CS does not directly affect the activation of CTL, even if proliferation is inhibited because IL-2 secretion by $CD4^+$ Th cells is still required. Similar results were obtained in a helper-deficient MLR (heat-treated stimulator cells) in which CTL generation occurs only when exogenous IL-2 is added to the cultures (121). In addition, CS (0.05 to 0.25 μg/ml) inhibits in a dose-dependent fashion the IL-2-driven proliferation of allo-activated human T cells in long-term cultures (39) or the activation of alloreactive CTL precursors in limiting dilution culture and in the presence of IL-2 (138, 177, 300, 411). CS (0.01 to 1.0 μg/ml) reduces CTL frequency by 90 to almost 100%, but a minor CS-resistant component has been observed both in humans and mice (138, 300). In limiting dilution cultures, CS blocks development of CTL generation but not cell proliferation of already activated cells (300).

CS prevents the generation of CTL in the MLR but does not affect the effector mechanism of mature CTL (37, 145, 154, 414). Activated CTL are largely resistant to the effect of CS and are also less sensitive to the effects of regulatory Ts cells than the naive precursor CTL (149, 355). After lymphocytes have been primed with antigen (48 to 72 h), the addition of CS has little or no effect on proliferation in response to rechallenge with the same antigen, but a new primary response to a second antigen is inhibited (184).

Heeg et al. (139) have analyzed the effect of IL-4 on the immune-suppressive action of CS during the in vitro primary activation of anti-MHC alloantigen-reactive

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CD8$^+$ CTL. IL-4 or IL-2 alone were unable to overcome the CS-mediated suppression (0.05 to 0.1 μg/ml), but the combined addition of exogenous IL-4 and IL-2 reversed the immune-suppressive effect of CS on the cytolytic activity of CD8$^+$ CTL, although the proliferative response remained depressed. The existance of a CS-resistant induction pathway for antigen-specific CD8$^+$ CTL may be of relevance for experimental and clinical organ transplantation. The fact that this CS-resistant pathway operates only in the presence of IL-2 and IL-4 suggests that IL-2 acts as a competence factor for IL-4. These data indicate that acquisition of cytolitic activity can be dissociated from T cell growth.

Another mechanism to explain the inhibitory effect of CS on MLR and CML could be induction of Ts cells (149, 210). These cells are radiosensitive (1500 rads), nylon-wool adherent, and cyclophosphamide sensitive, belong to the subpopulation defined by anti-CD8 MAbs, and express class II antigens (149, 152, 153). The interactions and cytokine requirements for Ts cells have not been clarified. Induction and amplification of Ts cells, in the presence or absence of CS, are dependent on the presence of CD4$^+$ Th cells (149, 347). Ts cells are detected at CS concentrations greater than 0.1 μg/ml (54), but at high doses of CS (1 μg/ml) inhibition of Ts cells results (34, 151, 152). The Ts precursor is not in itself blocked by CS, but effective suppressor induction requires IL-2. Reconstitution of these cultures with IL-2 restores the activation of the suppressor cells (34). The activation steps of Ts cells as well as their mechanism of action are not fully clarified, whether they act on Th cells or CTL precursors to limit the generation of cytotoxicity. A cytokine that causes Ts cell activation has been described and differentiated from IL-1, IL-2, IFN-α, IFN-β, and IFN-γ (336). It has also been reported that Ts cells release a genetically restricted factor with potent suppressive effects on a primary MLR (71). However, a complete characterization of these activities is still missing.

Inhibition of secondary MLR by CS (1 μg/ml) is mainly due to a block of IL-2 production (154).

Even if the functional significance of the autologous MLR is still a debated issue, it is considered an immunological response of antigen-specific T cells with receptors for self-MHC molecules to surface antigens present on autologous non-T cells (69). The class II MHC gene products are the major stimulating structures (208). Isolated CD4$^+$ but not CD8$^+$ T cells are capable of initiating the proliferative response; however, CD8$^+$ T cells do respond if CD4$^+$ T cells or IL-2 are present in the culture (339a). In the autologous MLR, CS inhibits both IL-2 production and the acquisition of IL-2 responsiveness (70). CS inhibits both primary and secondary autologous MLR only if the drug is added during the initial activation period but not 48 h later (306, 310). CS inhibits the induction but not the effector phase of CTL generation in the autologous MLR (306).

3. T Cell Clones and T Cell Lines

a. Introductory remarks. T cell clones, being homogeneous cell populations, provide useful information on CS activity. However, they are not representative of complex in vivo situations, because the influence of other cell types is absent, and they may acquire unique differentiation properties. Therefore, interpretation and extrapolation of data to the effect of CS on resting T cells must be approached cautiously. On the other hand, studies involving mixed populations of lymphocytes may give conflicting results, because there may be differential effects of the drug on individual cell populations (224), making it difficult to evaluate effects on specific cells or particular sites of drug action. In addition, the influence of antigen presentation and of accessory cells can be excluded in many experiments dealing with T cell clones. Human or murine CD4$^+$ Th clones, which proliferate and secrete lymphokines when stimulated with alloantigen, CD8$^+$ CTL clones, which proliferate to alloantigens only when exogenous IL-2 is provided, CD4$^+$ CTL clones, and antigen-independent but IL-2 dependent CTL clones have been used (135, 145, 301, 302). Few data are available on antigen-specific T cell clones.

Interactions between the APCs and the alloreactive Th cells or CTL can induce in the responding cell a variety of functions including proliferation, lymphokine production, and eventually lysis of the target cell. CS is very effective and already at doses of 0.01 μg/ml or lower causes significant inhibition of lymphokine secretion and proliferation (48, 302).

IL-2-producing T cell clones are heterogeneous with respect to their function and surface phenotype. Up to 20% of IL-2-producing clones coexpress cytolytic activity, and IL-2-producing cells belong to both CD4$^+$ and CD8$^+$ subsets (341). Generally, cytotoxic T cell lines are sensitive to IL-2 but not to the action of other factors such as IL-4 (89). Most CD8$^+$ clones that fail to secrete IL-2 produce IFN-γ after costimulation with antigen, APCs, and exogenous IL-2 (64).

b. Cell proliferation. The effect of CS on the proliferation of Th and CTL clones has been evaluated extensively. Proliferative responses induced by alloantigens in the presence or absence of IL-2, by anti-TCR/CD3 MAb, or by IL-2 alone have been studied. Most of the studies would substantiate the following conclusions: (1) Proliferation of alloantigen-stimulated Th clones of human or murine origin is highly sensitive to CS, mainly for the inhibition of IL-2 production (91, 145, 301, 302). The addition of excess IL-2 partially restores proliferation at low CS concentrations (0.01 μg/ml), whereas at higher CS concentrations (0.05 μg/ml) the inhibition of proliferation is only marginally affected (48). When helper T cell clones are cultured with specific alloantigen plus exogenous IL-2, additive proliferative responses are ob-

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served. Under these conditions, a CS-resistant component can be observed (48, 301). (2) The proliferative response of antigen-dependent IL-2-dependent CTL clones is sensitive to CS (0.1 μg/ml or less) (135, 301, 302, 315, 316), whereas antigen-independent IL-2-dependent alloreactive CTL, which represent the conventional CTL, are CS resistant (135, 145, 301, 302). The reactivation of $CD8^+$ CTL by alloantigen or by PMA, in the presence of IL-2, is inhibited at CS concentrations similar to those that block lymphokine transcription (0.02 to 0.20 μg/ml) (135). Additional evidence that CS affects the activation of CTL is the observation that, in limiting dilution cultures, the cloning efficiency and the frequency of cytotoxic clones are dramatically decreased (95%) despite the addition of excess exogenous IL-2 (39, 135, 300). (3) Proliferative responses of CTL to alloantigens or anti-TCR/CD3 MAb in which proliferation can occur via an IL-2-independent pathway have been described and are generally not affected by CS (145). (4) CS (0.03 μg/ml) inhibits proliferative response of antigen-specific and IL-4-producing murine Th2 clones stimulated by ionomycin and IL-1 or ionomycin plus PMA. Proliferative responses induced by IL-1 plus PMA are CS resistant (0.1 μg/ml), confirming that the stimulus is important in determining CS sensitivity or resistance. (5) Significant heterogeneity in the extent of inhibition and biphasic effects with higher levels of CS, producing inhibition not reversed by IL-2, have been observed in alloantigen-stimulated clones of different individuals or in different clones from the same individual (148, 182, 444). These diversified responses are independent from the specificity of the alloreactive T cell clones toward class I and II HLA antigens and of their belonging to the Th or CTL subset (444).

In conclusion these data suggest that CS inhibits proliferation in T cell clones in which the activation is mediated through a signal at the TCR/CD3 complex (first level of control), while the drug is less effective when the growth signal is delivered through a constitutively expressed IL-2*R* (second level of control) or through particular pathway of activation (160). In other terms, CS is more selective for resting T cells versus activated T cells.

c. Lymphokine production. The production of lymphokines by cloned Th or CTL is highly sensitive to CS inhibition. This effect is usually obtained with concentrations of CS lower than 0.1 μg/ml. The expression of IL-2, IFN-γ, GM-CSF, and IL-3 in $CD4^+$ Th clones, of IL-4 in murine Th2 clones, and of IFN-γ and macrophage-activating factor in $CD8^+$ CTL clones are depressed (145, 160, 302, 315, 422). In addition, CS inhibits both induced and ongoing production of IL-2 in antigen-stimulated T cell hybridomas (183). IL-2 synthesis in T cell lines (Jurkat human and EL-4 murine) activated by a lectin and/or PMA is also inhibited (80, 202, 220, 293, 433). However, CS does not inhibit IL-2 secretion by the human T lymphocyte line HUT 78 (248). In the murine T lymphoma cell line EL-4 and the human T-leukemia line Jurkat, CS at moderate concentrations (0.1 μg/ml) inhibits also the expression of IFN-γ and GM-CSF (359, 433). On the other hand, Bickel et al. (17, 18), who used the same murine lymphoma T cell line EL-4 and examined both specific mRNA production and lymphokine secretion, showed that CS (0.1 μg/ml) inhibits the secretion of IL-2 and IL-3 but not GM-CSF, suggesting that the production of these lymphokines are not directly linked. These discrepancies are difficult to reconcile and might indicate that different clones of the same cell line behave in a different fashion. These data are further evidence that CS does not simply block lymphokine gene expression but rather interferes with signaling pathways that bring to gene expression and T cell activation. Recently, a variant CTL clone that secretes IFN-γ after IL-2 stimulation has been isolated (75). In this particular clone, IFN-γ production induced by Con A is CS sensitive, whereas IFN-γ production induced by IL-2 is CS resistant. This observation is further proof that sensitivity of lymphokine gene expression to CS is related to the specific mode of stimulation and is not an obligate effect of CS (75).

Even if CS must be present from culture initiation to inhibit maximally lymphokine secretion in T cell clones, in some conditions a partial inhibition of mRNA can be expected even when lymphokine production has begun (145, 182). This observation has been reproduced in a human tumor cell line IARC 301 that constitutively secretes IL-2, expresses high affinity IL-2*R*, and requires IL-2 for growth (59). CS inhibits only the autocrine IL-2 synthesis and not the expression of high affinity IL-2*R* (59). The growth inhibition effect of CS (0.5 μg/ml) is reversed by addition of IL-2 at a concentration sufficient to saturate high affinity IL-2*R* (59). The finding that CS is able to inhibit an ongoing transcription of the IL-2 gene (118, 182) suggests that CS might have an effect on immune responses that have already initiated (145). However, this issue is controversial because other studies (202) suggest no inhibition of IL-2 synthesis if CS is added when IL-2 gene transcription has started.

Under conditions that inhibit lymphokine production by the cells, the expression of the TCR/CD3 complex, as assessed by the binding of specific MAbs, is not altered (145). In addition, the functional sites of the TCR are not affected by CS, as assessed by recognition and lysis of target cells by CTL (145, 235, 302).

d. IL-2R expression. Another point of controversy is the effect of CS on the expression of the IL-2*R* in cloned T cells. After the cell are activated and express high affinity IL-2*R*, the response of Th and CTL clones to exogenous IL-2 as well as the capacity to remove IL-2 from tissue culture medium appears insensitive to concentrations of CS that reduce the antigen-driven proliferation (0.5 μg/ml or more) (145, 302). This suggests

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that, in most cases, CS does not inhibit the expression of IL-2*R* in cloned T cells that constitutively express IL-2*R*. The same finding is characteristic of human T lymphocyte line Jurkat and IARC 301 which costitutively express low or high affinity IL-2*R* (59, 202, 359). However, in human $CD4^+$ cytotoxic T cell clone stimulated with the specific alloantigen (47) and in antigen-specific rat Th cells (46), the newly induced expression of both high affinity and low affinity IL-2*R* is inhibited by CS, even in the presence of exogenous IL-2 (50% inhibition in the presence of 0.5 μg/ml CS). This concentration is higher than that required to inhibit proliferation in the same clone (50% inhibitory concentration, 0.05 μg/ml in the presence of IL-2) (47).

e. Cytolytic activity. Lymphocytotoxity mediated by antigen-independent, IL-2-dependent CTL is insensitive to CS (145, 302), but lymphocytotoxicity mediated by antigen-dependent IL-2-dependent CTL and by antigen-dependent T cell hybridomas may be affected by CS (135, 182, 218). The maturation steps, which are a prerequisite for lytic activity, but not the cytolytic activity per se, are inhibited by CS (182). In CTL clones, CS does inhibit the expression of IFN-γ gene (145, 422) and of the genes that code for serine esterases (109, 215). Serine esterases are released during exocytosis and are involved in the lytic processes (142, 253, 383). Recently, Lancki et al. (218) have shown that CS inhibits exocytosis of serine esterase and CTL-mediated lysis of nonnucleated sheep red blood cell targeted by using anti-CD3 MAb. At the same doses, CS does not inhibit lysis of nucleated antigen-bearing target cells. Therefore, distinct effector mechanisms may be involved in the lytic activity of conventional $CD8^+$ CTL clones: (1) a CS-sensitive mechanism that correlates with exocytosis and is independent of damage to target cell nuclei and (2) a CS-insensitive mechanism that does not require exocytosis and that requires the presence of a nucleus in the target cell (218).

In addition, a partial direct effect of CS on regeneration of cytotoxic activity in CTL, which are antigen dependent and IL-2 dependent for activation, has been reported (135). Such an effect seems unrelated to suppression of IL-2 production and is unexpected, because most studies have not described this phenomenon (148). However, the alloantigen-dependent CTL used in this study differ from the alloantigen-independent CTL which have been used in most studies, and this may explain the different results (135).

Recently, antigen-specific MHC class II-restricted CTL or inflammatory cells have been reported (390, 391). These cells are able to lyse nonspecific bystander target cells in the presence of their specific target (390, 391). These inflammatory $CD4^+$ T cell clones require IL-2 to acquire the nonspecific cytolytic activity necessary to lyse third-party cells (125). CS, by inhibiting IL-2 secretion, can block bystander cell lysis (125). The capacity to lyse a variety of bystander cells could be an important function of $CD4^+$ CTL clones. A role for these subsets in the rejection of kidney transplants could be envisaged as well as an additional inhibitory effect for CS.

D. NK Cells and LAK Cells

Cell-mediated killing is another important function of the immune system that has been studied in relation to CS. NK cells, a heterogeneous population of cells with lytic activity, have been implicated in defense against malignancies (143). Human NK cells are well characterized and are primarily large granular lymphocytes (389). The cell line K562 is the prototype of the human NK target. The recognition receptor expressed by NK cells is not identical with the TCR/CD3 complex, its molecular nature remains elusive, and it does not appear to recognize MHC molecules (144). IL-2 stimulates NK cell activity directly and perhaps indirectly as well, by stimulating production of IFN-γ (87, 317, 419). NK cell-mediated cytotoxicity is potentiated by all classes of IFN, and NK cells themselves can produce IFN-γ (87). In spite of a few controversial results, most studies show that CS does not significantly influence NK cells in humans and rodents. In the human MLR, CS does not prevent an increase in NK activity, and it does not influence the increase of NK activity induced by IFN-γ or IL-2 (121, 219, 409). However, an indirect effect of CS through the block of IL-2 and IFN-γ secretion by T cells is possible. The slight decrease of NK activity in CS-treated MLR may reflect an impairment of IL-2 or IFN-γ production (121, 219). Introna et al. (167) found that CS (10 μg/ml) inhibited NK activity only when the lymphocytes were incubated for 20 h prior to assay. A few controversial data have been reported. In the mouse, Tanaka et al. (384) reported that CS treatment had little effect on NK cell activity, whereas Yanagihara and Adler (438, 439) showed that CS (1.5 μg/ml) suppressed NK cells. This effect was rapid and did not require T cells, B cells, macrophages, or suppressor cells.

LAK precursors are neither mature T cells nor NK cells, but they do express a minimal number of IL-2*R*, which increase in parallel with the development of a polyspecific cytolytic activity (123). Proliferation is essential for the activation of LAK cells, and exogenous IL-2 activates LAK cells. Their lytic activity is MHC unrestricted (147). CS (1 μg/ml) only partially inhibits induction of LAK cells (121, 123). LAK precursors can be functionally differentiated from allospecific CTL precursors, and CS may affect this population of cells through inhibition of lymphokine secretion.

E. B Cells

1. Introductory Remarks

B cells are heterogeneous in their response to a variety of stimuli (170). Regulation of B cell responsiveness is complex because many factors play a role (163). Several lymphokines produced by Th cells are involved in the

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activation, clonal expansion, differentiation, and isotype regulation of B cell subsets. IL-2 can directly stimulate B cell proliferation and differentiation in both human and murine systems (283, 399). IFN-γ can enhance B cell differentiation (232) and IgG2a secretion of polyclonally activated B cells (366). IL-1, IL-4, IL-5, IL-6, TNF, and lymphotoxin are involved at various levels in B cell responses (158, 188, 282, 376, 410).

CS inhibits B cell responses that depend on lymphokine secretion by T cells. In addition, CS may have a direct inhibitory effect on some B cell subsets. In the mouse, some B cell populations appear to be CS sensitive and other CS resistant (196). However, CS may suppress or enhance B cell activation, depending on the stimulus triggering the cells (62). Different polyclonal B cell activators can stimulate the same B cell subsets through distinct intracellular pathway (196, 281). CS inhibits the activation of resting B cells by agents causing rapid breakdown of phosphatidylinositol biphosphate and mobilization of intracellular $[Ca^{2+}]_i$, such as anti-Ig antibody or calcium ionophores, and is much less effective on responses to activators which do not provoke $[Ca^{2+}]_i$ mobilization such as IL-4, LPS, or PMA (19, 20, 197, 198). CS does not interfere with $[Ca^{2+}]_i$ mobilization but rather with a later event along this pathway (19).

Activated B cells, like T cells, bear IL-2*R* (Tac) (239, 399). CS has no effect on the induction of IL-2*R* and transferrin receptors on LPS-induced murine B cells (235). Data concerning the expression of IL-2*R* (Tac) by other stimuli or of other lymphokine receptors are missing. In the mouse, the increase in MHC class II antigen expression induced by PMA is CS resistant, whereas that induced by calcium ionophores is CS sensitive (197). CS (0.5 μg/ml) causes an early acceleration of the capping of mouse B cell membrane Ig (276).

TNF is a lymphokine that is secreted also by B cells (375). TNF and lymphotoxin, which is closely related to TNF in structure and function, enhance the proliferation of SAC-activated tonsillar B cells (188, 189). CS inhibits the direct effect of SAC and the synergistic effects of PMA with anti-μ or SAC to increase TNF mRNA expression in B cells but has no effect on the stimulation by PMA alone (375).

With one exception (murine anti-μ-stimulated B cells), the susceptibility of B cell activation to CS wanes rapidly with time after stimulation. CS has a selective inhibitory effect on the activation phase and is less effective during the proliferative and differentiative phases. Species differences in the sensitivity to CS have been described (196).

2. *Human B Cells*

All polyclonal B cell activators except for Epstein-Barr virus (EBV) require T cells or T cell-derived factors for the generation of Ig-secreting cells in man, although some induce B cell proliferation in a T cell-independent manner (171).

a. PWM. PWM is a T cell- and monocyte-dependent polyclonal B cell activator (413) that stimulates a subset of preactivated B cells (212). $CD4^+$ Th cells, which secrete B cell growth and differentiation factors, are required for Ig synthesis (335), whereas $CD8^+$ T cells are involved in suppressor circuits (387). IL-2 is involved in B cell activation and differentiation (284). Human PBMC, cultured in the presence of CS (1 μg/ml), fail to proliferate and to produce Ig in response to PWM (14, 22, 284, 303, 392). CS must be present throughout the culture period and has no effect on Ig synthesis when added to the cultures at day 4 or later (304). Interindividual variations in the sensitivity to CS have been observed (303). Coculture experiments demonstrate that a functional inactivation of Th cells can explain the inhibitory effect of CS (392). Ig production is substantially reconstituted by the addition of IL-2 at the beginning of the culture, and the kinetics of inhibition of Ig production and of IL-2 secretion by CS are closely related (284). The IL-2 effect is dependent on the presence of PWM, suggesting that at least two signals are required for Ig production. These data do not exclude the involvement of other lymphokines. Most studies indicate an indirect effect of CS on B cells; however, a direct effect of CS on PWM-reactive B cells has been proposed (14, 133).

b. SAC, anti-μ antibody and other CS-sensitive stimuli. Several studies suggest that CS suppresses proliferation and Ig synthesis of human PBMC stimulated with SAC (14, 133, 281, 303) and the proliferative response of human B cells stimulated with anti-μ antibody in the presence or absence of growth factors (14, 281). The response of B cells to costimulation with anti-μ antibody and growth factors is 50- to 100-fold more sensitive to CS than SAC-induced B cell proliferation (281).

SAC-induced proliferation is T cell independent, suggesting that B cells are directly affected by CS. However, because Ig secretion is T cell dependent (348) and IFN-γ synergizes with IL-2 in the differentiation of SAC-activated B cells (284), the inhibitory effect of CS on Ig secretion could be explained by its action on T cells. CS must be added also in this case during the initial culture period (281, 304).

Proliferation, cell enlargement, and RNA synthesis of small resting B cells triggered by anti-μ antibody are inhibited by CS (281). The inhibitory effect is evident only when the drug is added within 24 h of culture initiation. After small B cells are activated, the cells become resistant to CS (1 μg/ml) and proliferate in the presence of growth factors. In humans, CS selectively suppresses an early step of B cell activation and has little inhibitory effect on the subsequent factor-dependent proliferation and differentiation (281).

In addition, the T cell-dependent polyclonal B cell activation of PBMC stimulated with a soluble extract of

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ultrasonically disrupted mycobacteria is also inhibited by CS (98).

c. EBV. EBV activates B cells in the absence of monocytes, T cells, or T cell factors (21). In human PBMC, EBV-induced B cell proliferation and Ig secretion are resistant or are enhanced by CS (1 μg/ml) (14, 22, 62, 392). The amount of EBV added and the source of lymphocytes may influence the effect of CS on Ig secretion (168). The enhancement of the response to EBV in the presence of CS is due to the fact that CS prevents the activation of EBV-specific Ts cells which interfere with B cell activation (252, 392). However, in vivo CS has no inhibitory effect on Ts cells present in the peripheral blood of patients with EBV-induced infectious mononucleosis (392).

CS facilitates the in vitro outgrowth of B-lymphoblastoid cell lines from lymphocytes of EBV-immune donors (22, 56, 67, 307). CS specifically deletes or functionally inactivates immune T cells (22). CS strongly inhibits the proliferation of T cells induced by EBV-infected autologous cells and the generation of Ts cells and CTL against EBV-infected autologous cells (307).

d. Spontaneous Ig production. CS does not inhibit the spontaneous antibody secretion by circulating B cells obtained after in vivo immunization. Brieva and Stevens (31), who used CS at the concentration of 1 μg/ml, did not observe any effect on the spontaneous antibody production by PBMC collected 5 and 7 days after immunization with tetanus toxoid. Munoz and Insel (280) showed that, after immunization with *Haemophilus influenzae* type b capsular polysaccharide and tetanus toxoid, CS (0.2 μg/ml) enhanced spontaneous antibody secretion. These different results could be explained by the higher dose of CS used by Brieva and Stevens (31). These data confirm that spontaneous in vitro antibody production is controlled by T cells and that CS abolishes a suppressive mechanism. Similarly, spontaneous IgE synthesis is not affected and sometimes is enhanced by CS (0.5 μg/ml) (350).

3. B Cells of Other Species

In the mouse, B cell responses can be induced by T cell-independent antigens (TI) and T cell-dependent antigens (TD). TD require T cell help to permit B cell differentiation into antibody-secreting cells. TD are normally sensitive to CS (29). TI have been further subdivided into two classes based on the ability of TI stimuli to activate B cells from the CBA/N mouse strain (2). Thus, TI-1 stimuli such as LPS can stimulate B cells from CBA/N mice (271), whereas TI-2 stimuli such as Ficoll and dextran do not (277). CS impairs the response to TI-2 and enhances that to TI-1 (207).

In vitro a variety of mitogenic and nonmitogenic polyclonal activators have been tested (196). The latter group includes agents such as intact (IgG) rabbit anti-Ig antibody, Con A, PMA, calcium ionophores, and IL-4 which cause abortive B cell activation, i.e., they drive the cells into a transitional activation state (G_1 phase) but do not induce proliferation (20). These stimuli can be classified as being either CS sensitive (calcium ionophores or Con A) or CS resistant (IL-4 and PMA) (196). There is a correlation between the capacity to mobilize $[Ca^{2+}]_i$ and CS sensitivity (196).

a. Anti-Ig and other CS-sensitive stimuli. In vitro CS (0.01 μg/ml) preferentially blocks the response of B cells to dextran sulfate (157), anti-Ig antibody (68), and calcium ionophores (197) or to these stimuli in combination with phorbol esters (20).

Almost all B cells can be driven into the G_1 phase of the cell cycle by anti-Ig antibody, and a portion progresses to proliferation (61). CS (0.01 μg/ml) blocks murine B cell activation by anti-IgM antibody which is a monocyte- and T cell-independent stimulus (68, 207). CS suppresses this proliferative response at 300- to 400-fold lower concentrations than those required to inhibit B cell proliferation induced by LPS (68, 296). Anti-μ-stimulated B cell proliferation remains susceptible to inhibition by CS for 36 to 48 h. This represents an exception to the rule that susceptibility to CS in culture wanes rapidly with time (68, 296). However, when B cells are driven to proliferation by submitogenic doses of anti-Ig and LPS, CS sensitivity lasts only for a few hours after activation (68). Proliferation of B cells induced by submitogenic doses of anti-Ig antibodies and IL-4 is CS sensitive (296).

In contrast, White et al. (426) found that CS failed to inhibit the proliferation of porcine B cells to anti-pig IgM. Species differences may account for these controversial results.

In the mouse, the proliferative responses to dextran sulfate (157) or to the combination of calcium ionophores plus PMA are inhibited by low concentrations of CS (296).

IL-5 acts as a growth and differentiation factor for large B cells preactivated by anti-Ig in vitro (295). The stimulation of naturally occurring large B cells by IL-5 to synthesize DNA or to secrete Ig is extremely sensitive to CS (0.01 μg/ml) (296). IL-5 does not cause $[Ca^{2+}]_i$ mobilization and represents an exception to the rule that CS affects the $[Ca^{2+}]_i$-dependent pathway. These results suggest that, in the mouse, CS affects all stages of B cell stimulation (196) and contrast with the human situation in which B cell proliferation and Ig secretion induced by T cell-derived lymphokines are CS resistant (281). This may represent a difference between murine and human B cells. However, because recombinant lymphokines were not used, and a mixture of lymphokines were actually investigated, other explanations remain possible. Further studies are required to define more precisely these points.

b. LPS and other CS-resistant stimuli. CS has much less effect on responses to activators that do not provoke $[Ca^{2+}]_i$ mobilization such as LPS or PMA (19, 197, 198).

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LPS stimulates a distinct activation pathway in B cells. CS (1 μg/ml) does not inhibit LPS-induced proliferation and Ig secretion in murine splenocytes (29, 374).

Similar to the in vivo situation, enhanced antibody responses to the TI-1, dinitrophenyl-LPS are demonstrable in vitro in the presence of CS (157). Antibody production in vitro is enhanced at the same CS concentrations that inhibits proliferation by 40 to 50% (157). The enhancement is not removed by T cell depletion and might result from a direct action of the drug on the responding B cells (157).

Activation of B cells into G_1 by PMA is CS resistant (198). Phorbol esters synergize with LPS, and RNA synthesis in B cells stimulated by these two agents is not affected by CS (20). The activation of resting murine B cells by IL-4 is CS resistant, whereas B cell proliferation elicited by costimulation with anti-Ig plus IL-4 remains drug susceptible (296).

c. Spontaneous Ig secretion. Under conditions of high cell density and in the absence of any stimulus, mouse spleen cells produce after 7 days of culture IgM, IgG, IgM anti-DNA, and IgG anti-DNA (255, 322). In mice, spontaneous in vitro production of anti-DNA Ig by autoimmune MLR-lpr/lpr mice, by autoimmune NZB mice, or by control BALB/c mice (255, 322) is inhibited by CS. Total Ig production is inhibited, but the synthesis of IgM and IgG anti-DNA is 10- to 100-fold more sensitive to the inhibitory activity of CS (255). Anti-DNA production in vitro by spleen cells stimulated with LPS is CS resistant (255). Functional studies (137) indicate that Ly-1^+ B cells are responsible for the production of most of the autoantibodies, whereas B cells in the Ly-1^- lineage participate in the conventional responses to foreign antigens (137).

In a system in which a persistent antibody response is established, CS inhibits spontaneous formation of anti-ovalbumin IgE antibodies (298). CS might activate Ts cells that suppress the spontaneous antibody-forming cells (298).

F. Effect of CS on Other Systems

1. Hematopoiesis

Studies in experimental systems, animals, and humans suggest that T lymphocytes may be involved in regulating hematopoiesis. T cells can interact with hematopoietic stem cells by several mechanisms, including direct cell-cell contact or the release of growth factors or lymphokines, which affect hematopoiesis. Synthesis of IL-3, GM-CSF, and erythroid burst-promoting activity are examples of positive regulation of hematopoiesis, whereas synthesis of IFN-γ may inhibit hematopoiesis. However, the observation that CS inhibits secretion of IL-3, GM-CSF, and other hematopoietically active lymphokines by T cells, but does not affect hemopoiesis, may indicate that the role of T cells in normal hematopoiesis is limited and that other cell types may produce them.

No significant inhibition of human colony-forming unit-culture (granulocytes) was observed at concentrations of CS up to 1 μg/ml; inhibition was observed at 10 μg/ml (114, 141). Enhancement of human colony-forming unit-culture was observed by another group in the presence of T cells (100). These data suggest that CS may inhibit an endogenous, T cell-mediated, suppressor mechanism. CS at concentrations exceeding 2 μg/ml shows a dose-dependent suppressive effect on colony-forming unit-culture (100). Lennon et al. (234) showed that CS indirectly inhibited erythroid colony formation in dogs, presumably by interfering with CS-sensitive accessory cells.

CS does not interfere with chemotaxis, oxidative metabolism, and microbial activity of human neutrophils and thus does not perturbate the major defense system against most infections. In human neutrophils, CS (10 μg/ml) has no effect on arachidonic acid release and metabolism (290) and on oxidative burst, chemotaxis, and bactericidal activity against *S. aureus* and *Pseudomonas aeruginosa* (29, 192, 420). However, inhibition of chemotaxis toward formylmethionyl-leucyl-phenylalanine has been reported (72, 73). Polymorphonuclear leukocytes from renal transplant recipients receiving CS and prednisone demonstrate normal metabolic burst and bacterial killing (420).

2. Tumor Cell Lines

CS has a possible cytostatic effect on malignant cells. The inhibitory effect of CS on the proliferation of certain malignant cell lines of lymphoid, hematopoietic, and nonhematopoietic origin has been reported (13, 94, 115, 259, 322, 351, 393, 440). Mainly T cell but also some B cell tumor cell lines are sensitive to the suppressive effect of CS (1 μg/ml) (94, 115, 322, 393). CS may also inhibit malignant squamous cell lines (241). In conclusion, this drug has some antiproliferative activity mainly directed to T cells (440).

Inibition of lymphokine synthesis and secretion needed for growth, interference with intracellular nucleotide levels, and mitochondrial effects are all possible events induced by CS that may affect cellular division (94). Some observations suggest that CS may prevent DNA synthesis by inhibition of ornithine decarboxylase (106). In the murine thymic leukemia cell line EL4, CS treatment has an inhibitory effect on ornithine decarboxylase, the initial enzyme in the polyamine cascade necessary for DNA synthesis (92). Indeed, the inhibition by CS of the growth of rodent pancreatic or colon cells in vitro can be blocked by the addition of polyamine (351, 352).

The development of drug resistance by tumor cells is a major obstacle to the cure of human malignancy. CS abrogates pleiotropic drug resistance in certain experimental tumors (258, 363).

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3. Other Systems

The effect of CS on keratinocytes may be relevant to evaluate whether keratinocytes are a cellular target for the action of CS in persons with psoriasis and other skin diseases. Some studies showed that CS directly inhibits the growth of keratinocytes in culture (101, 288), whereas Kato et al. (180), who used pig keratinocytes, did not observe such an effect. An antiproliferative effect was observed at doses of 0.3 to 1.0 μg/ml when the cultures were prolonged for 6 days (101). As already mentioned, higher levels of CS are found in the skin than in blood, and these levels may be considered pharmacological levels.

The arterial response to injury is characterized by proliferation of smooth muscle cells. CS causes a persistent inhibition of intimal cell proliferation through an effect on the immune system; CS has no direct effect on smooth muscle proliferation in culture (174). No effect of CS on cultured human endothelial cells was observed (395). However, CS (0.1 to 2.0 μg/ml) inhibits the growth of rat glomerular mesangial cells (250) and of pig kidney tubule cells (10).

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