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Functional Differentiation of Human Cytotoxic T Lymphocytes in the Presence of FK 506 and CyA

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THE recognition of antigen is the first step in maturation of effector T lymphocytes. Gromo et al have shown that antigen can trigger a precursor T lymphocyte to respond to additional signals necessary to induce full functional maturation.[1,2] Using minimal signals, such as MoAbs to CD2 receptor or MoAbs directed against CD28, they have demonstrated that precursor cytotoxic T lymphocytes (pTcs) can differentiate into pre-effector cytotoxic cells (peTcs). Following the addition of recombinant IL-2 (rIL-2) or IFN-γ, peTcs will acquire cytolytic activity.[1,2] Likewise, stimulation with the calcium (Ca^{2+}) ionophore A23187 drives pTcs into the peTc stage, and rIL-2 induces peTcs to become effector cytolytic cells (eTcs).[1]

CyA, a T cell-specific immunosuppressive drug, inhibits the generation of effector cells by blocking IL-2 and, to a lesser degree, IL-1 production (reviewed in reference 3). A new immunosuppressive drug, FK 506, has properties similar to CyA, but it is 100 to 400 times more potent.[4-9] The strong in vitro inhibitory effect of FK 506 can be demonstrated in primary mixed lymphocyte cultures (MLRs) and secondary proliferation of alloreactive T cells propagated from organ transplant biopsies.[6-9]

CyA and FK 506 appear to inhibit the transcription of early T cell activation genes, including those coding for IL-2, IL-3, and IFN-γ.[3,10,11] These drugs do not affect the proliferation of activated T cells in the presence of IL-2 or the cytolytic effector function.[7,8]

Little is known of the effect of these drugs on the stages of cytolytic T lymphocyte (CTL) generation. This study was designed to evaluate the effects of these drugs on the stepwise activation, proliferation, and maturation of CTLs induced by A23187 and rIL-2.

MATERIALS AND METHODS

Cells

PBLs were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal healthy donors.[7] Cells were resuspended in tissue culture medium (TCM) consisting of RPMI 1640 supplemented with 25 mmol/L Hepes buffer and 100 U/ml gentamicin, with 10% normal AB human serum. Viability of isolated PBLs was routinely 99% as determined by staining with 1% eosin.

Drug Sources

CyA was obtained from Sandoz (Basel, Switzerland) and was dissolved in ethanol (1 mg/ml). FK 506 was kindly provided by Fujisawa Pharmaceuticals (Osaka, Japan) as a crystalline powder and was dissolved in methanol (1 mg/ml). rIL-2 (Sandoz) was obtained as a stock solution of 100,000 U/ml. A23187 (Sigma, St Louis, MO) was diluted in ethanol (1 mg/ml). Phytohemglutinin (PHA), concanavalin A (Con A) and phorbol myristate acetate (PMA) were obtained from Sigma and used at 1%, 50 μg/ml, and 0.1 μg/ml, respectively.

Proliferation Under Different Activation Conditions

Triplicate cultures of PBL were set up in TCM at 2×10^6/ml in the presence of A23187 (0.75-1.0 μg/ml), PHA (1%), or PMA (0.1 μg/ml) in a volume of 200 μl for 3 days. Proliferation was assessed by ^{3}H-thymidine uptake during the last 20 hours of incubation, harvested onto fiber glass paper, and counted in a liquid scintillation counter (LKB).

Dose Effects of FK 506 and CyA on Lymphocyte Proliferation

The inhibitory effects of CyA and FK 506 on the lymphocyte response to different activation conditions were assessed. The concentrations used were 1 and 0.1 ng/ml of FK 506 and 100 and 10 ng/ml of CyA. The results were expressed as the percent of inhibition of the proliferative response using the following formula:

$$\% \text{ inhibition} = (1 - \text{cpm with drug/cpm without drug}) \times 100$$

^{51}Cr Release

Cytotoxicity was determined in a standard LDCC assay as previously described.[2] LDCC was performed in the presence of Con A at 50 μg/ml. The effector to target ratio was 20:1. The percentage of lysis was calculated using the following formula:

$$\% \text{ lysis} = (\text{experimental} - \text{background/total} - \text{background}) \times 100$$

The effectors were harvested at day 3 after initiation of the culture for the first phase of activation, and after an additional 2 days of culture in the presence of 100 U/ml of rIL-2 for the second phase of activation. The RPMI cell line was used as the target in LDCC assays.

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Table 1. Inhibitory Effects of FK 506 and CyA on the Response Induced by A23187, PHA, and PMA

Mitogen	Proliferation* (cpm ± SD)	Percent Inhibition of the Proliferative Responses			
		FK 506 (1 ng/ml)	FK 506 (0.1 ng/ml)	CyA (100 ng/ml)	CyA (10 ng/ml)
A23187 (0.75 μg/ml)	19,979 ± 2,240	98	67	99	78
A23187 (1 μg/ml)	34,077 ± 6,181	95	33	97	51
PHA (1%)	180,465 ± 9,558	53	28	48	16
PMA (0.1 μg/ml)	104,806 ± 8,826	1	3	2	1

*Background proliferation: 286 ± 124 cpm.

IL-2 Production

IL-2 was measured in a bioassay using an IL-2-dependent T cell line, CTLL-2.[12] Supernatants were collected after 24 hours under each activation condition in the presence or absence of immunosuppressive drugs. CTLL-2 cells were plated at 10^4/well and 100 μl/well of supernatant in a 96-well flat-bottomed plate. Triplicate cultures were incubated for 24 hours and pulsed with 1 μCi/well of ^{3}H-thymidine during the last 8 hours. rIL-2 was used as a positive control.

RESULTS

Effects of FK 506 and CyA on the Proliferative Responses of Human Peripheral Blood Lymphocytes

The proliferative response of PBL induced by A23187 was significant but less than PHA or PMA stimulation (Table 1). FK 506 and CyA inhibited over 90% of the response to A23187 at doses of 1 ng/ml and 100 ng/ml, respectively. PHA-induced proliferation of PBL was less sensitive than A23187-stimulated proliferative response, and at doses of 1 ng/ml FK 506 or 100 ng/ml CyA, only 50% inhibition was achieved. Furthermore, the mitogenic response of human PBL to PMA was completely resistant to FK 506 or CyA (Table 1). These results indicated that FK 506 significantly inhibited proliferative responses to A23187 and PHA, and this effect was observed at doses 100-fold lower than with CyA.

Effect of FK 506 and CyA on IL-2 Secretion

The secretion of IL-2 at 24 hours after stimulation with A23187 was inhibited by FK 506 (1 ng/ml) and CyA (100 ng/ml), whereas PMA-induced IL-2 release was not affected (Table 2). IL-2 release following A23187 stimulation was more inhibited by low doses of FK 506 and CyA (0.1 ng/ml and 10 ng/ml, respectively) than was PHA-induced IL-2 release.

Effects of FK 506 and CyA on the Maturation of Cytotoxic T Cells

A23187-activated cells had no detectable LDCC activity (Table 3), whereas PHA activation induced a significant LDCC response (24%). PMA-stimulated cells exhibited intermediate LDCC activity (12%). On addition of rIL-2 (100 U/ml) for 2 days, all cultures demonstrated significant cytolytic activity (Table 3). Addition of FK 506 and CyA during this second step did not affect the development of cytotoxic activity in A23187-stimulated cells nor did it inhibit the augmented response of PHA-activated cells. Thus, neither FK 506 nor CyA influenced the IL-2-induced conversion of peTcs to CTLs.

DISCUSSION

The present study demonstrates that FK 506 and CyA significantly inhibit the proliferation and IL-2 release of A23187-stimulated cells but have no effect on the maturation of peTc to active CTL when help is provided in the form of rIL-2. Gromo et al have shown that the induction of CTL involves a series of events including proliferation and differentiation.[1,2] To study the effect of immunosuppressive drugs on the various stages, we employed the stepwise approach using minimal signals that do not, by themselves, result in full maturation to effector cytolytic cells. An example of a minimal signal associated with activation along alternative pathways is A23187-induced stimulation of pTcs to peTcs. These peTcs can proliferate but do not exhibit CTL activity.

A23187-induced proliferation is IL-2-dependent, and low doses of FK 506 or CyA can fully suppress the proliferation and IL-2 release of these cell cultures. The concept of IL-2 dependence of A23187 induction of T cell proliferation is controversial. Recently, Chatila et al dem-

Table 2. Inhibitory Effects of CyA and FK 506 on IL-2 Release by Activated T Lymphocytes

Mitogen	CTLL-2* Proliferation (cpm ± SD)	Percent Inhibition of IL-2 Release			
		FK 506 (1 ng/ml)	FK 506 (0.1 ng/ml)	CyA (100 ng/ml)	CyA (10 ng/ml)
A23187 (0.75 μg/ml)	21,065 ± 4,272	99	88	99	96
A23187 (1 μg/ml)	73,092 ± 1,148	99	26	99	51
PHA (1%)	31,009 ± 1,199	67	6	70	23
PMA (0.1 μg/ml)	38,598 ± 715	1	3	2	1

*^{3}H-thymidine incorporation of the IL-2-dependent CTLL-2 cell line in the presence of supernatants obtained 24 hours after stimulation in different culture conditions. Background proliferation: 67 ± 18 cpm. Proliferation in the presence of 100 U/ml IL-2: 110,358 ± 6,822 cpm.

Table 3. Cytotoxic Activity of PBL Stimulated With A23187, PHA, and PMA

Mitogen	% CML* (No Drugs)	% CML* After Addition of Exogenous IL-2 (100 U/ml)		
		No Drugs	FK 506 (1 ng/ml)	CyA (100 ng/ml)
A23187 (0.75 μg/ml)	3 ± 1	56 ± 1	60 ± 3	67 ± 3
A23187 (1 μg/ml)	3 ± 1	64 ± 6	65 ± 8	67 ± 3
PHA (1%)	24 ± 3	43 ± 1	36 ± 3	48 ± 2
PMA (100 ng/ml)	12 ± 1	39 ± 2	n/t	n/t

*The effector to target ratio was 20:1.
n/t denotes not tested.

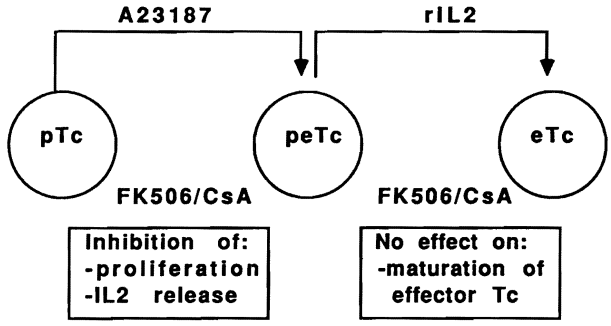

Fig 1. Effects of FK 506 and CyA on proliferation and functional maturation of human cytotoxic T cells.

onstrated that the Ca^{2+} ionophore, A23187, is a monocyte-dependent T cell mitogen associated with IL-2 production and that it is partially inhibited by anti-IL-2 receptor MoAb.[13] On the other hand, several laboratories have previously reported IL-2-independent induction of T cell proliferation by A23187.[14,15] The discrepancies among various laboratories may be related to the experimental conditions and, in particular, to the choice of Ca^{2+} ionophores.

Confirming previous reports,[1,2,15] we have observed that A23187 alone cannot drive the pTc to an eTc. Cells responding to an antigen or anti-CD3 MoAb release IL-2 and express cytotoxic activity, whereas those stimulated by the minimal signals do not produce detectable IL-2 and fail to exhibit cytolytic activity.[2] On the other hand, our findings suggest that A23187-stimulated cells do not mature to CTLs in spite of detectable IL-2 release. It is possible that the level of IL-2 accumulated in A23187 cultures was not enough to provide the second signal, or that A23187-stimulated cells exhibited a low affinity to the IL-2 released in these cultures. IL-2 production of A23187 and PHA-stimulated cells was in the same range. PMA activation induced comparable levels of IL-2, also without inducing significant CTL activity. These findings suggest that under our experimental conditions, A23187 and PMA may deliver more than one type of signal to T cells. Gromo et al proposed at least three categories of signals in T cell activation.[1] The "minimal signal" may drive the differentiation of pTcs to peTcs, the "inhibitory signal" apparently prevents cells from proceeding further in the maturation pathway, and the "bi-functional signals" can deliver both a positive and a negative signal. A23187 stimulation induces maturation of pTcs to peTcs, but prevents the peTcs from progressing into eTcs. Likewise, PMA and other molecules may also deliver more than one type of signal, depending on dose, time of exposure, and other factors.[16]

Lymphocyte activation by A23187 was more sensitive to inhibition by FK 506 and CyA than was activation by PHA. Kay et al have also reported that A23187 activation of pig lymphocytes is more sensitive to CyA inhibition than Con A stimulation.[17]

In vitro studies with Ca^{2+} ionophore and phorbol esters have shown that increased concentrations of Ca^{2+} and activation of protein kinase C appear to be required for the full activation of T cells.[3] CyA interferes with certain pathways that stimulate gene expression in T cells induced by Ca^{2+} but not with those stimulated by protein kinase C.[3] Likewise, FK 506 affects A23187 activation events but has no effect on PMA-induced activation and IL-2 gene expression.

Another response resistant to immunosuppressive drugs FK 506 and CyA is the IL-2-driven proliferation of activated T cells.[7-9] Similarly, IL-2-driven maturation of peTcs is not inhibited by FK 506 or CyA. Thus, these drugs affect only the pTc → peTc stage but not the peTc → eTc stage (Fig 1). Recently, two groups have shown that CyA and FK 506 bind to different proteins (cyclophilin and FK 506 binding protein) that share a common enzymatic property.[18,19] Both proteins have isomerase activity that may be important in Ca^{2+}-dependent intracellular signalling events in T cell activation.[18,19]

FK 506 induces considerable prolongation of allograft survival in various species: cardiac transplants in rats, renal and liver allografts in dogs, and kidney allografts in baboons.[20-23] Delayed treatment with FK 506 (ie, on days 5, 6, and 7 posttransplant) was also shown to be effective in preventing allograft failure in several experimental models.[22,23] The in vivo findings do not contradict our in vitro observations. Allograft rejection is a complex process in which lymphokines and activated effector cells all play important roles. FK 506 is a potent drug that efficiently suppresses lymphokine production, thus shutting off ongoing allograft rejection. FK 506 is currently being used at our institution in phase I clinical trials to treat "rescue" patients who have failed to respond to conventional therapy[24] and as the primary immunosuppressive agent in liver transplantation.[25]

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