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Relative Roles of T-Cell Receptor Ligands and Interleukin-2 in Driving T-Cell Proliferation

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Abstract Stimulation of T cells by the T-cell receptor (TCR)/CD3 complex results in interleukin-2 (IL-2) synthesis and surface expression of the IL-2 receptor (IL-2R), which in turn drive T-cell proliferation. However, the significance of the requirement of IL-2 in driving T-cell proliferation, when TCR stimulation itself delivers potential mitogenic signals, is unclear. We show that blocking of IL-2 synthesis by Cyclosporin A (CsA) suppressed both the Concanavalin A (Con A)- and phorbol myristate acetate (PMA)/ionomycin-induced proliferation of T cells. The latter is also inhibited by anti-IL-2R. Kinetic studies showed that T-cell proliferation begins to become resistant to CsA inhibition by about 12 h and became largely resistant by 18 h of stimulation. PMA, the protein kinase C activator, enhanced Con A-induced T-cell proliferation if added only within first 12 h of stimulation, and not after that. Given the fact that, in the present study, TCR is downregulated within 2 h of Con A stimulation and T cells entered the S phase of cell cycle by about 18 h of stimulation, the above results suggest that TCR stimulation provides the initial trigger to the resting T cells, which allows the cells to traverse the first two third portions of G1 phase of cell cycle and become proliferation competent. IL-2 action begins afterward, delivering the actual proliferation signal(s), allowing the cells to traverse the rest of G1 phase and enter the S phase of the cell cycle. J. Cell. Biochem. 76:37–43, 1999.

Key words: IL-2; Cyclosporin A; T-cell receptor; T-cell proliferation

Stimulation of T cells through the T-cell receptor (TCR)/CD3 complex initiates a cascade of early biochemical events, the hallmarks of which are the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the activation of diacylglycerol (DAG)/Ca^{2+}-dependent protein kinase C (PKC) [for review, see Hadden, 1988]. These two events initiate remainder of the cascade of events that constitute the T-cell activation, manifested in clonal expansion (proliferation) and functional differentiation of T cells. Among the several physiological changes in T cells brought about by the activated PKC and increased $[Ca^{2+}]_i$, the milestones are interleukin-2 (IL-2) secretion and IL-2 receptor (IL-2R) expression, which are obligatory for T-cell proliferation stimulated through the TCR/CD3 complex [for review, see Isakov et al., 1987a]. In fact, it has been shown that IL-2 is apparently the only requirement for T-cell proliferation after IL-2R is expressed [Konaka, et al., 1981]. IL-2 action begins with its binding to IL-2R, followed by the activation of signaling cascade(s) involving multiple nonreceptor protein tyrosine kinases and DAG/Ca^{2+}-independent isotypes of PKC [for review see Rebollo et al., 1996].

Despite the absolute requirement of IL-2 for T-cell proliferation and several works on the IL-2 signaling mechanisms, it is unknown why IL-2 is required for T-cell proliferation when antigenic/mitogenic stimulation through TCR/CD3 complex delivers potential mitogenic signals, as in nonlymphoid cells a single mitogen, many of which lead to activation of PKC and increased $[Ca^{2+}]_i$, is sufficient to induce proliferation without the involvement of any additional growth factor [for review, see Pazin and Williams, 1992]. Thus, we investigated the relative contribution of TCR ligand and IL-2 in driving the proliferation of resting T cells. Our results showed that TCR signaling can provide the initial trigger, involving DAG/Ca^{2+}-sensitive PKC and increased $[Ca^{2+}]_i$, to the T cells to traverse from the resting state through first two third portions of G1 phase of cell cycle but not beyond that. IL-2 action begins after that

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pushing the cells through remainder of G1 into the S-phase (DNA synthesis phase) of cell cycle.

MATERIALS AND METHODS

Reagents

Concanavalin A (Con A) was purchased from Pharmacia AB (Uppsala, Sweden). RPMI-1640 was from HiMedia Laboratories Limited (Mumbai, India). Ionomycin was purchased from Calbiochem-Novabiochem International (San Diego, CA). [^{3}H]thymidine (spec act 18,000 mCi/mmole) was from Bhabha Atomic Research Center (Mumbai, India). Phorbol 12-myristate 13-acetate (PMA), L-glutamine and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Cyclosporin A (CsA) was purchased from Cipla Pharmaceutical Co. Fetal bovine serum (FBS) was purchased from Gibco-BRL Life Technologies (Grand Island, NY). Monoclonal antibodies to mouse IL-2 receptor were obtained from Boehringer-Mannheim (Indianapolis, IN).

Cells and Culture Medium

T cells were isolated from the spleen of Balb/C mice in RPMI-1640 medium supplemented with 2 mM L-glutamine, streptomycin (0.1 mg/ml) and Gentamycin (0.2 mg/ml) as described by Chakrabarti et al. [1995]. Unless otherwise stated, isolated T cells were maintained and cultured in above RPMI-1640 supplemented with 10% heat-inactivated FBS (complete medium) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation Assay

The proliferation of T cells was measured by the incorporation of [^{3}H]thymidine in the replicating DNA. T cells (1×10^5 cells/well in 0.1 ml) were stimulated with Con A or a combination of PMA and ionomycin in a round-bottom 96-well plate. The cells were pulsed with [^{3}H]thymidine (1 µCi/ml) at 24 h, harvested with a PHD cell harvester at 60 h of culture; [^{3}H]thymidine incorporation was measured by a liquid scintillation counter. The effect of various agents on proliferation was assessed by comparing the proliferation in the presence of the agent with control proliferation and expressed as follows: the percentage of control proliferation = 100 × proliferation in the presence of the agent/control proliferation (proliferation in the absence of the agent).

Determination of Cell Viability

After treatment with various agents, the viability of T cells was measured by MTT assay [Mosmann, 1983]. T cells were washed and incubated with 2.5 mg/ml MTT in complete RPMI medium for 4 h at 37°C in a 5% CO_2 incubator. After the cells were washed with normal saline, the formazone crystals formed were solubilized in isopropanol containing 0.04 N HCl. The absorbency of the solution was measured at 492 nm in an E_{max} automated enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices).

RESULTS AND DISCUSSION

We first investigated the inhibition of Con A-induced T-cell proliferation by CsA, which inhibits IL-2 gene transcription by inhibiting Ca^{2+}/calmodulin-regulated phosphatase calcineurin after binding to its intracellular receptor cyclophillin [Clipston and Crabtree, 1992]. T cells were stimulated with the maximum effective dose of Con A (10µg/ml) in the presence or absence of various concentrations of CsA. As expected, the addition of CsA at the beginning of the culture suppressed Con A-induced T-cell proliferation in a dose-dependent manner (Fig. 1). The inhibition started at a concentration of 0.025 µM and became maximized at 0.25 µM CsA. The cell viability was not affected at any concentration of CsA used (data not shown). These results confirmed the inhibitory effect of CsA on mitogen-induced T-cell proliferation.

Activation of PKC and increased $[Ca^{2+}]_i$ are the key TCR signaling events. These two events initiate the remaining events, including IL-2 synthesis and IL-2R expression, leading to T-cell proliferation [Hadden, 1988; Truneh et al., 1985]. Therefore, direct activation of PKC with a phorbol ester and increased $[Ca^{2+}]_i$ with a Ca^{2+} ionophore can lead to IL-2 synthesis, IL-2R expression, and finally T-cell proliferation, bypassing the requirement of TCR stimulation [Truneh et al., 1985; Berry et al., 1990]. However, involvement of IL-2 in phorbol ester/Ca^{2+} ionophore-induced T-cell proliferation is not clearly understood. Although the activation of PKC and increase in $[Ca^{2+}]_i$ are key TCR signaling events in T-cell proliferation, TCR/CD3 complex is downregulated very rapidly (within 2 h) after interacting with its ligand [Valitutti et al.,

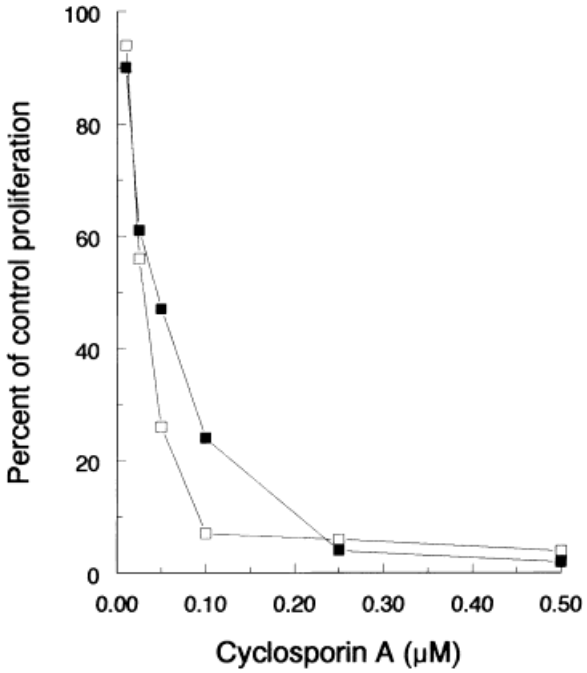

Fig. 1. Effect of Cyclosporin A (CsA) on Concanavalin A (Con A)-induced T-cell proliferation. Freshly isolated T cells (1×10^5/well) were stimulated to proliferate with 10 μg/ml Con A in 96-well plates. Various concentrations of CsA were added to the culture at the beginning of stimulation. Cells were pulsed with [^{3}H]thymidine (1 μCi/ml) at 24 h and harvested by a PHD cell harvester at 60 h of culture. [^{3}H]thymidine incorporation (cpm) was measured by a scintillation counter. The percentage of control proliferation was calculated as 100 × proliferation in the presence of the agent/proliferation in the absence of the agent (control proliferation). Results of two experiments are presented and each point is the average of duplicates.

1995], as we also found that prior stimulation for 2 h with Con A rendered the T cells unresponsive to further stimulation with the same dose of the mitogen (Fig. 2A). By contrast, a long period of stimulation is required for the proliferation of freshly isolated resting (naive) T cells, which are in the G0/G1 phase of cell cycle [Chakrabarti and Engleman, 1991], in response to a mitogenic stimulus. In the present study, we observed that murine T cells (1) entered the S phage of the cell cycle about 18 h after stimulation with Con A (Fig. 2B), and (2) did not proliferate if Con A and other secreted factors were removed from the culture even after 8 h of stimulation (Fig. 2C). Thus, the appearance of IL-2/IL-2R system, upon TCR stimulation, functions to save TCR-initiated events from being futile and lead to T-cell proliferation.

There may be two ways of IL-2 functioning in T-cell proliferation. First, IL-2 may be required to maintain the mitogenic events, initiated by TCR stimulation for sufficient period of time leading to T-cell proliferation. Second, TCR signaling events may provide an initial drive to the resting T cells, making them proliferation competent, including IL-2 production and IL-2R expression. IL-2 then acts on the competent cells and delivers the actual proliferation signal. To distinguish between these two possibilities, we assessed whether IL-2 is required in T-cell proliferation induced by direct activation of PKC and increase in $[Ca^{2+}]_i$ with a phorbol ester and a Ca^{2+} ionophore. If the first possibility is true CsA should not inhibit the proliferation, as phorbol esters and Ca^{2+} ionophore maintain the PKC translocation/activation and increase in $[Ca^{2+}]_i$ for sufficiently longer period of time to induce proliferation [Truneh et al., 1985; Rando, 1988; Berry et al., 1990; Asaoka et al., 1991]. Just the reverse will happen if the second possibility is true. Thus, T cells were stimulated with a combination of maximum mitogenic concentrations of PMA (0.25 nM) and ionomycin (2 μM) in the presence or absence of various concentrations of CsA. Our results showed that CsA suppressed the PMA/ionomycin-induced proliferation in a dose-dependent manner without affecting cell viability in the same way as suppressing Con A-induced proliferation. Inhibition was detectable at 0.025 μM and became maximum with 0.25 μM CsA (Fig. 3A). It is possible that, unlike inhibiting the mitogen/antigen-induced proliferation, CsA might inhibit PMA/ionomycin-induced proliferation nonspecifically; this result was corroborated by our finding that anti-IL-2R antibody also inhibited the PMA/ionomycin-induced proliferation in a dose-dependent manner (Fig. 3B). The variation between experiments 1 and 2, depicted in Figure 3B, can be attributed to the known individual variation in the expression of IL-2R on the T cell surface after stimulation. These results clearly showed that IL-2 is required in T-cell proliferation stimulated by activating PKC and increasing $[Ca^{2+}]_i$ directly, suggesting that IL-2 functions to deliver the actual proliferation signals to the T cells rendered competent when subjected to TCR stimulation.

The above results contradict the previous finding that CsA and αIL-2R antibody did not inhibit proliferation of human T cells stimulated with PKC activators such as Teleocidine, 12-O-tetradecanoylphorbol 13-acetate (TPA), and PMA [Isakov et al., 1985a, 1987a;

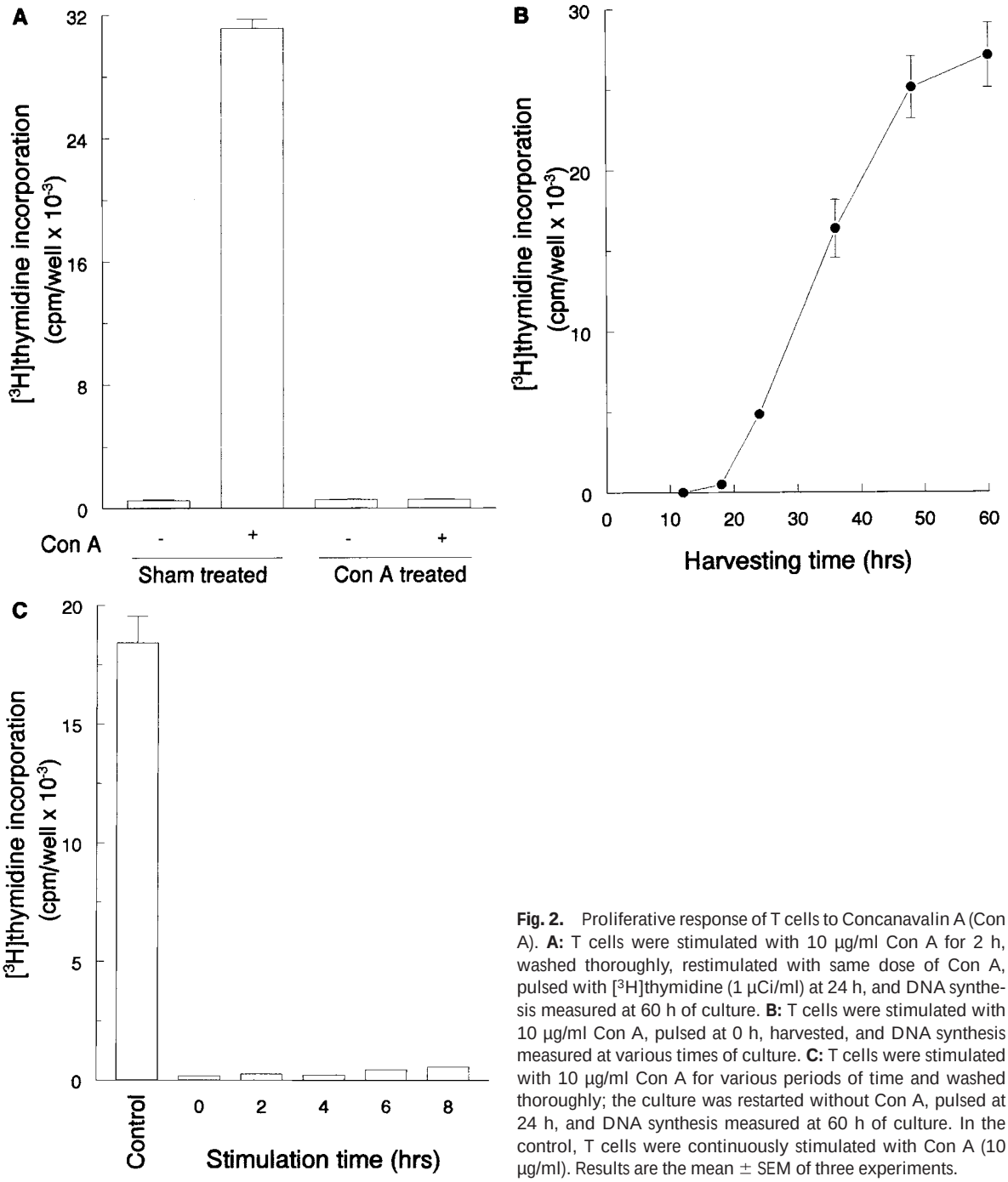

Fig. 2. Proliferative response of T cells to Concanavalin A (Con A). **A:** T cells were stimulated with 10 μg/ml Con A for 2 h, washed thoroughly, restimulated with same dose of Con A, pulsed with [^{3}H]thymidine (1 μCi/ml) at 24 h, and DNA synthesis measured at 60 h of culture. **B:** T cells were stimulated with 10 μg/ml Con A, pulsed at 0 h, harvested, and DNA synthesis measured at various times of culture. **C:** T cells were stimulated with 10 μg/ml Con A for various periods of time and washed thoroughly; the culture was restarted without Con A, pulsed at 24 h, and DNA synthesis measured at 60 h of culture. In the control, T cells were continuously stimulated with Con A (10 μg/ml). Results are the mean ± SEM of three experiments.

Bloemena et al., 1989]. However, it should be pointed out that these agents can induce human T-cell proliferation without IL-2 production [Isakov et al., 1985a,b, 1987b; Bloemena et al., 1989]. Also, in murine T cells, TPA fails to induce IL-2 synthesis, even in the presence of the Ca^{2+} ionophore and is not mitogenic [Koyasu et al., 1987]. We also observed that PMA alone did not induce proliferation in mouse T cells even at very high concentration (data not shown). Thus, a species-specific IL-2-independent mitogenic effect of phorbol ester could account for the lack of any effect of CsA and αIL-2R antibody on the proliferation induced by these agents in human T cells. Finally, our results are in agreement with a previous find-

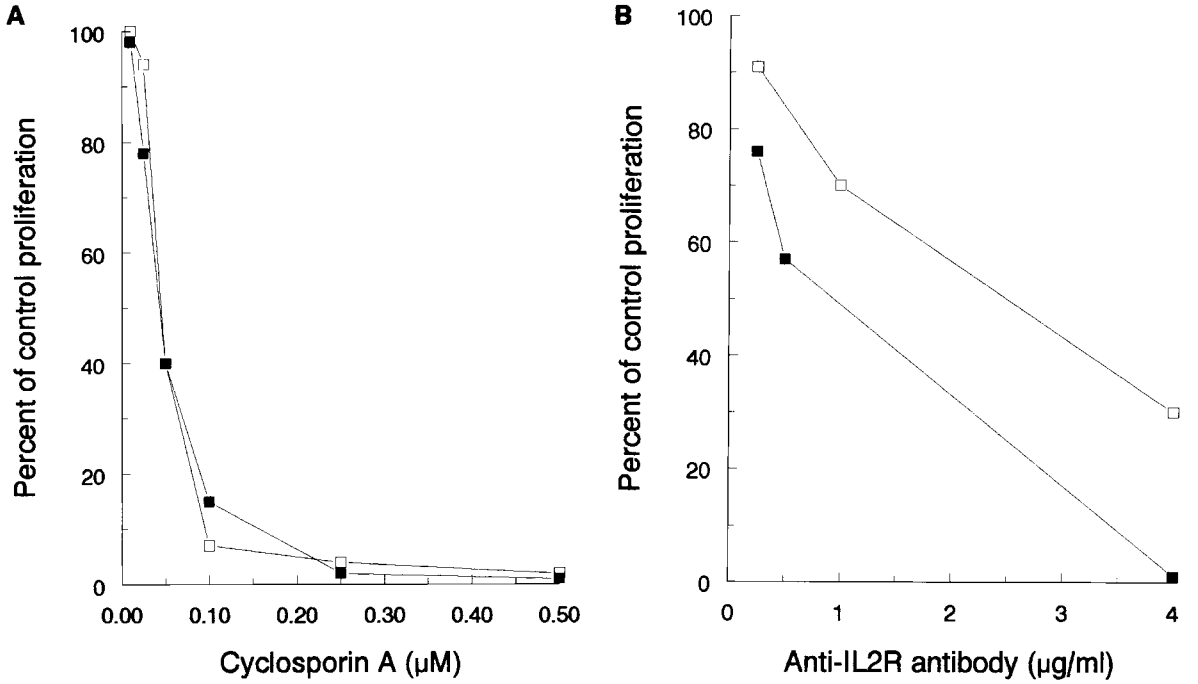

Fig. 3. Effect of Cyclosporin A (CsA) and anti-IL-2R antibody on phorbol myristate acetate (PMA)/ionomycin-induced T-cell proliferation. Freshly isolated T cells were stimulated to proliferate with a combination of PMA (0.25 nM) and ionomycin (2.0 μM) in the presence or absence of various doses of CsA **(A)** and anti-interleukin-2 receptor (IL-2R) antibody **(B)** as in Fig. 1. Results of two experiments are presented; each point is the average of duplicates.

ing that PMA/A23187-induced proliferation of murine T cells is inhibited by CsA and anti-IL-2R [Nobrega et al., 1986].

Downregulation of TCR complex within 2 h of stimulation may not necessarily lead to the simultaneous downregulation of TCR signaling events and the beginning of IL-2 action. Thus, we sought to determine the point at which the activity of the TCR signaling events is over and IL-2 action begins to drive T-cell proliferation. Toward that end, T cells were stimulated with Con A, and CsA was added at various times of stimulation. Our results showed that cells started to become refractory to CsA by 12 h of culture and became largely refractory by 18 h of culture (Fig. 4). In the present study, T cell DNA synthesis (S phase) begins at about 18 h and is completed by 48 h of culture (Fig. 2B). This finding suggests that IL-2 action began roughly by the end of the first two third portions of G1 phase and is completed roughly when cells enter the S-phase of the cell cycle. This is compatible with a report indicating that, in mouse splenocyte culture, IL-2 utilization begins by 12 h of stimulation with Con A [Gelfand et al., 1987]. Why, then, did the cells fail to become completely refractory to CsA at 18 h of stimulation? This is because the asynchronous nature of the cells allows the majority—but not all—of the cells in a population to pass a given phase of cell cycle at a given time,—in the present study, 18 h to enter into the S-phase. Therefore, a slow increase in refractoriness to CsA was observed even after 18 h of stimulation. To complement this finding, we explored the period during which TCR signaling events are required to drive T cell activation. We therefore examined the temporal pattern of the enhancing effect of PMA on Con A-induced T-cell proliferation, as we found that PMA enhanced Con A-induced T-cell proliferation (data not shown). Because PMA/DAG-sensitive PKC is not involved in IL-2 action, enhancement of Con A-induced proliferation by PMA would indicate the active state of TCR signaling events. The temporal pattern of the effect of PMA on Con A-induced proliferation showed that the addition of 20 nM PMA within first 12 h of stimulation resulted in 126% and 78% enhancement in proliferation, respectively (Fig. 4). However, the addition of PMA after 12 h failed to enhance proliferation. On the basis of these

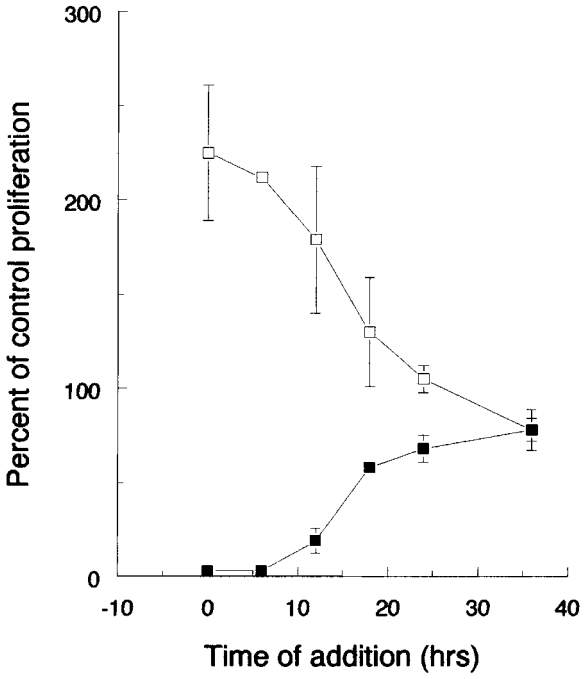

Fig. 4. Temporal patterns of the effect of Cyclosporin A (CsA) and phorbol myristate acetate (PMA) on Concanavalin A (Con A)-induced T-cell proliferation. T cells were stimulated to proliferate with 10 μg/ml Con A as in Fig. 1; 0.25 μM CsA (solid square) and 20 nM PMA (open square) were added at different times of stimulation. Results are the mean ±SEM of three experiments.

results, it appears that IL-2 action begins roughly when the functions of TCR signaling events are over.

This article provides an insight into the relative contribution of TCR and IL-2 signaling events in driving T-cell proliferation. Based on the results presented, we propose that TCR signaling provides the initial trigger to the resting T cell driving it through the first two third portions of G1 phase (first 12 h) of cell cycle, and not beyond that even TCR signaling events are maintained. IL-2 action begins after that and delivers the actual proliferation signal allowing the cells to enter the S-phase of the cell cycle.

Finally, the question arises regarding the necessity of the IL-2/IL-2R system to drive T-cell proliferation, when TCR stimulation itself delivers potential mitogenic signals. Based on the present and several other findings, we provide the following explanation in the light of fine-tuning of the specific immune response to an antigen. An extremely small number of the peptide-major histocompatibility complex (MHC) (antigenic complex) is available to activate a T-cell clone [Harding and Unanue, 1990; Demotz et al., 1990]. To compensate for the scarcity of antigen, the interaction of TCR with the antigenic complex has high off-rate [Weber et al., 1992; Matsui et al., 1991; Corr et al., 1994], which enables the small number of antigenic complex to engage and trigger a large number of TCRs serially [Valitutti et al., 1995]. However, the interaction of antigenic complex, at any concentration, with TCR results in the rapid downregulation of the latter [Valitutti et al., 1995]. As prolonged stimulation is required for the proliferation response, the above properties of antigenic complex-TCR interaction would enable extremely small amount of antigen to activate a large number of T cells of a given clone, but can not ensure the rapid expansion of that clone to launch an effective immune response. Thus, T cells have evolved another system, which remains dormant in resting T cells, but is upregulated in activated cells and drives clonal expansion. This system is the IL-2/IL-2R system. In a nutshell, it can be said conceivably that this dual system has been evolved to ensure the specific selection of antigen-specific clones by a small amount of antigens and rapid expansion of only that selected clone to mount an effective and specific immune response.

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