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The Regulation of T-Cell Proliferation: A Role for Protein Kinase C

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A. Introduction

T-lymphocyte proliferation is regulated by the T-cell growth factor interleukin 2 (IL-2) which exerts its biological effects through an interaction with high-affinity specific IL-2 receptors [1–3]. Quiescent T-lymphocytes neither produce IL-2 nor express IL-2 receptors [2, 4]. However, following immune stimulation there is transcriptional activation of both the IL-2 and IL-2 receptor genes which results in IL-2 synthesis and IL-2 receptor expression [5–7]. T-cell proliferation can then proceed via an autocrine pathway in which the population secretes and responds to its own growth factor.

Recently there have been considerable advances in our understanding of the signals that initiate IL-2 production and IL-2 receptor expression. In particular, the T-cell membrane structures involved in antigen recognition and the associated immune activation have been identified. The T-cell antigen receptor is an idiotypic disulphide-linked heterodimer (Ti) comprising two glycosylated polypeptides (α and β) of M_r 50 000 and 43 000 respectively [8–11]. Ti is associated noncovalently on the cell surface with the invariant T3 antigen [8, 12]. T3 consists of three chains [13, 14] – two glycosylated polypeptides of M_r 26 000 and 21 000 (γ and δ) respectively and one non-N-glycosylated peptide of M_r 19 000 (ε) – and is generally considered to be involved in the intracellular transduction of the signals that initiate T-cell growth [15, 16]. The nature of these intracellular signals has not been defined although it has been proposed that the T3/Ti complex is linked to a phosphodiesterase that metabolizes phosphatidylinositol and generates two potential intracellular signals, inositol triphosphate and diacylglycerol [17]. Inositol triphosphate is thought to mobilize intracellular calcium and thus elevate intracellular Ca^{2+} concentrations whereas diacylglycerol has been linked to activation of a calcium/phospholipid dependent kinase, protein kinase C [18]. In this respect, calcium ionophores which elevate intracellular Ca^{2+} concentrations and phorbol esters which stimulate protein kinase C can mimic the effect of immune activation and initiate T-cell proliferation via the IL-2 system [19].

An interesting feature of the T cell is that IL-2 production and IL-2 receptor expression are both transient [4–7]. For example, the polyclonal activation of T-lymphocytes induces a short phase (3–4 days) of autocrine proliferation followed by a prolonged phase (10–14 days) in which the cells are responsive to an exogenous supply of IL-2 [4]. These proliferative characteristics reflect the fact that initially there is induction of both IL-2 production and IL-2 receptor expression which then drives T-cell proliferation in an autocrine system. IL-2 production is switched off rapidly, which is why autocrine proliferation ceases, whereas IL-2 receptor expression and hence IL-2 responsiveness declines more slowly over the 10–14 day period. These unique characteristics of the T-cell proliferative system have focused obvious questions as to the molecular events that determine the transient nature of IL-2

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production and IL-2 receptor expression, since these ensure the homeostasis of the T-cell proliferative response.

In the present report we have used phorbol esters to explore the role of protein kinase C in the regulation of T-cell proliferation. Our data show that protein kinase C may have a dual role in the T cell since activation of protein kinase C can deliver positive signals crucial for the initiation of IL-2 production and IL-2 receptor expression. As well, activation of protein kinase C can deliver negative signals to the T cell and induce unresponsiveness with respect to the initiation of T-cell proliferation via triggering of the T3/T cell antigen receptor complex. Consequently protein kinase C may determine the transient nature of the T-cell growth response.

B. The Role of Protein Kinase C as a Positive Growth-Regulatory Signal in the T Cell

Quiescent T-lymphocytes can be activated by monoclonal antibodies that recognize the T3 antigen and trigger the T3/T-cell antigen receptor complex [20–22]. For optimal induction of T-cell proliferation there is an obligate requirement for monocytes/macrophages as accessory cells. This monocyte requirement can be substituted by phorbol esters which activate protein kinase C. As well, T3/Ti triggering can be substituted by calcium ionophores which increase the concentration of cytoplasmic free calcium [23].

To compare the effect of these stimuli, either singly or in combination, on the induction of IL-2 production and IL-2 receptor expression, we examined the initiation of T-cell growth in the presence or absence of exogenous IL-2. The induction of autocrine T-cell proliferation requires optimal induction of both IL-2 receptor expression and IL-2 production. However when IL-2 is nonlimiting (e.g. provided exogenously in excess) the T-cell proliferative response is a direct measure of the cellular density of high-affinity IL-2 receptors. Consequently, a comparison of the signals is necessary to induce autocrine T-cell proliferation versus IL-2 responsiveness allows a rapid comparison of the signals that induce IL-2 receptor expression and IL-2 production. For such studies we have chosen to examine the secondary stimulation of T-lymphocytes that have been arrested in the G_0/G_1 stage of the cell cycle by prior activation and clonal expansion in IL-2. After 10–14 days of culture such cells assume the phenotype of a quiescent T-cell population and have the advantage of giving a synchronous response to activation [4]. Additionally it is possible to obtain large numbers (10^9) of T cells with no contaminating accessory cells present.

The data in Fig. 1 are derived from an experiment in which quiescent T-lymphocytes were exposed to various combinations of the anti-T3 antibody OKT3, phorbol 12,13 dibutyrate (Pdbu), the calcium ionophore, ionomycin, and IL-2. A combination of OKT3 plus Pdbu (Fig. 1 a, c) or ionomycin plus Pdbu (Fig. 1 b, c) could stimulate autocrine T-cell proliferation whereas the various stimuli given singly or the combination of OKT3 plus ionomycin were ineffective. In contrast, a single stimulation with Pdbu or OKT3 could induce IL-2 responsiveness (Fig. 1 a, c); ionomycin had no effect. These results suggest that a single stimulus of T3/Ti triggering or activation of protein kinase C by Pdbu is sufficient to induce IL-2 receptor expression and hence IL-2 responsiveness. In contrast, a combined stimulus of T3/Ti triggering plus protein kinase C activation or calcium ionophore plus protein kinase C activation is necessary to ensure both IL-2 receptor expression and IL-2 production and hence allow an autocrine proliferative response.

C. The Role of Protein Kinase C as a Negative Growth-Regulatory Signal in the T Cell

Activation of protein kinase C by Pdbu induces T cells to become responsive to IL-2 (Fig. 1 c). However, Pdbu-activated T cells are refractory to proliferative signals delivered via the T3/Ti complex by anti-T3 antibodies (Fig. 2). Since Pdbu-activated T cells can respond to IL-2 (Fig. 1 c), it is probable that the lack of a growth response to anti-T3 reflects an inhibition of T3/Ti-in-

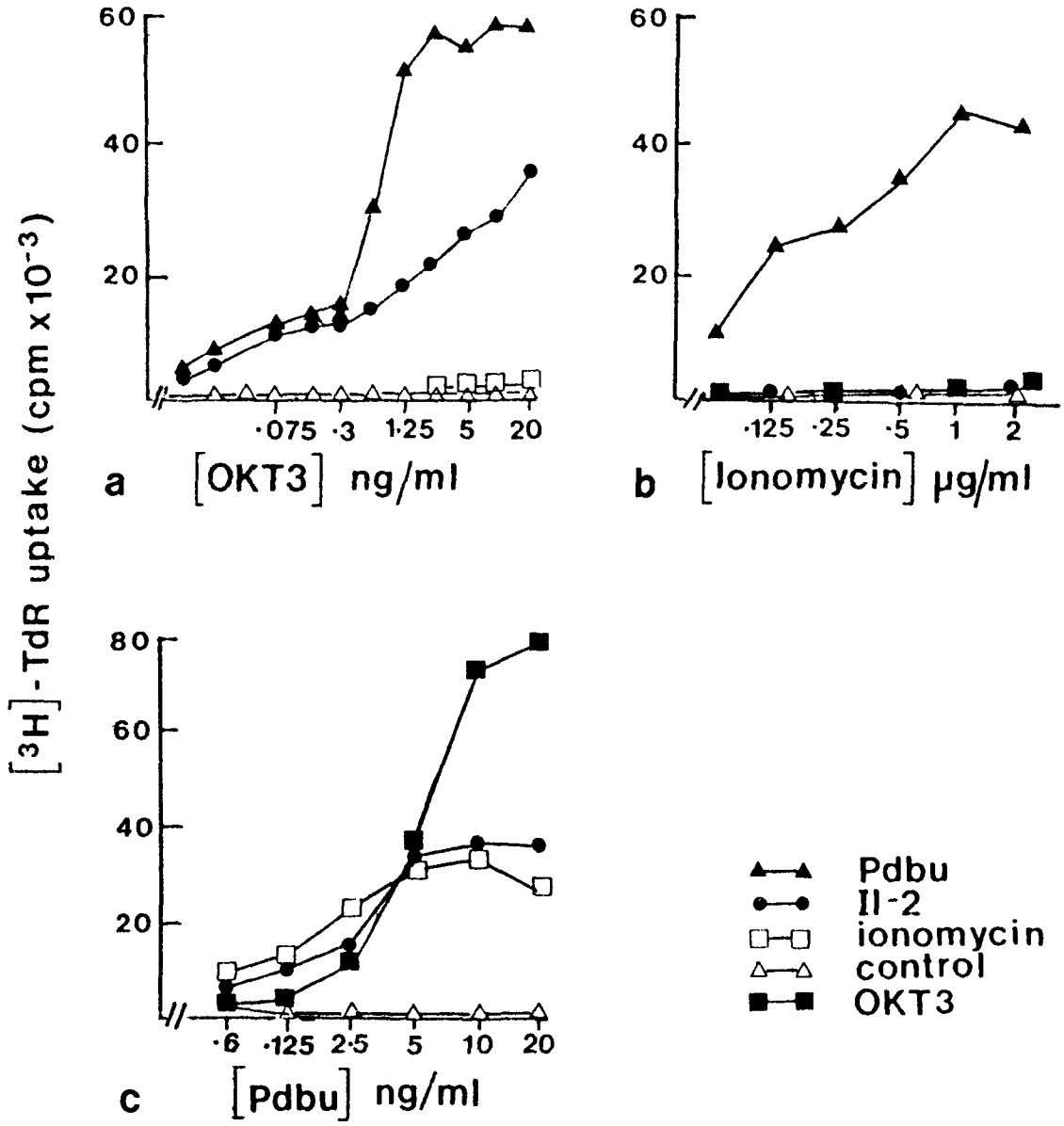

Fig. 1 a–c. Human T cells were prepared as described previously [4, 26]. Briefly, peripheral blood derived T cells were activated polyclonally by incubation for 72 h in RPMI 1640 supplemented with 10% FCS and 1 ng/ml OKT3 (Ortho Pharmaceuticals). Cells were maintained during activation at 37 °C in a humidified 5% CO_2/air incubator. Thereafter cells were maintained at 10^5–10^6/ml in the presence of 1 unit/ml recombinant IL-2 for 10–14 days, after which the cells became quiescent and did not produce IL-2 or express IL-2 receptors. For restimulation 10^5 cells/well were cultured for 48 h in microtest II plates in a final volume of 200 µl in the presence of the activating signals described below. Tritiated thymidine (^{3}H-TdR) incorporation (0.5 µCi/ml) was monitored over a 4-h period as an estimate of DNA synthesis. Data are shown as ^{3}H-TdR uptake (cpm/10^5 cells). **a** Cells were exposed to various concentrations of OKT3 (0–20 ng/ml) alone (△–△) or in the presence of 5 ng/ml Pdbu (▲–▲), 1 unit/ml IL-2 (●–●), or 0.5 µg/ml ionomycin (□–□). **b** Cells were exposed to various concentrations of ionomycin (0–4 µg/ml) either alone (△–△) or in the presence of 5 ng/ml OKT3 (■–■), 1 unit/ml IL-2 (●–●), or 5 ng/ml Pdbu (▲–▲). **c** Cells were exposed to various concentrations of Pdbu (0–50 ng/ml) either alone (△–△) or in the presence of 5 ng/ml OKT3 (■–■), 1 unit/ml IL-2 (●–●), or 0.5 µg/ml ionomycin (□–□)

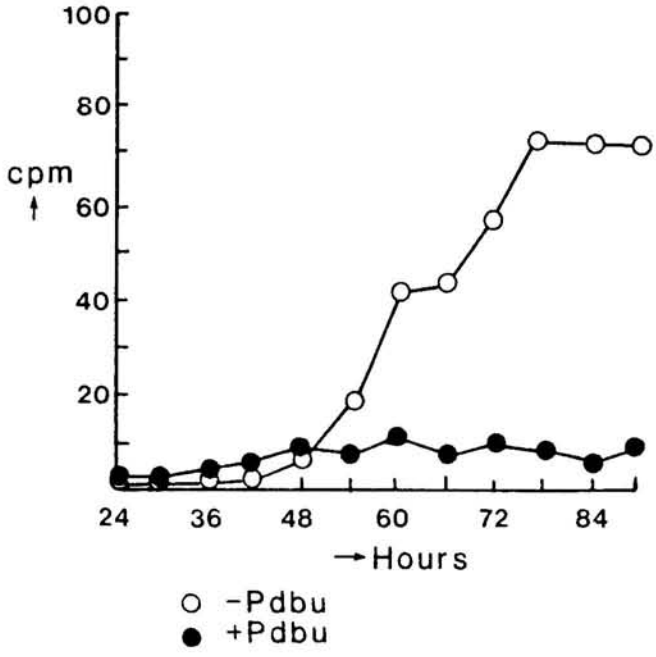

Fig. 2. Human peripheral blood derived T-lymphocytes and monocytes were purified by adherence to fibronectin-coated dishes and passage over nylon wool columns (G. Dougherty and H. Hogg, personal communication). Monocytes were cultured for 40 h at 10^4 cells/well in microtest II plates. T cells were cultured for 40 h either in the presence or absence of 50 ng/ml Pdbu, after which cells were washed three times with RPMI 1640/10% FCS. Control (o–o) and Pdbu-treated cells (●–●) were then exposed to 5 ng/ml OKT3 in the presence or absence of monocytes. The data show ^{3}H-TdR uptake (cpm/10^5 cells) (2-h pulse) in cells cultured for 90 h at 10^5/well in the presence of 10^4 monocytes/well in a final volume of 200 µl. No detectable ^{3}H-TdR uptake was detected in T cells exposed to OKT3 in the absence of monocytes or in T cells cultured with monocytes alone

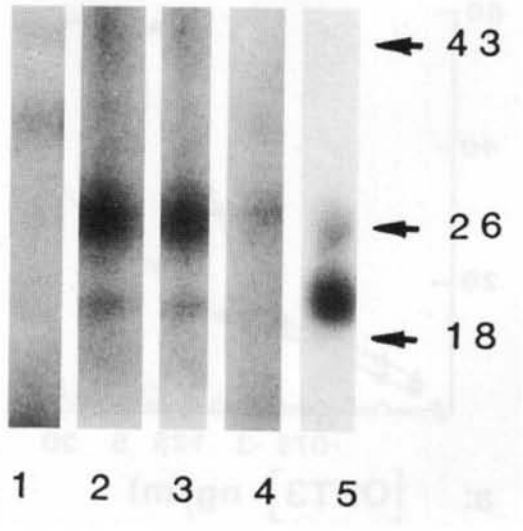

Fig. 3. ^{32}P-labelled peripheral blood derived lymphocytes were treated with PHA for 0, 5, 15, 40 min (*tracks 1, 2, 3, 4* respectively) prior to immunoprecipitation with UCHT1, a monoclonal antibody against the T3 antigen.
Methods: Human peripheral blood mononuclear cells from a single donor were isolated by Ficoll-Hypaque discontinuous gradient centrifugation and cultured 10^6/ml for 18 h in phosphate-free Eagle's medium, supplemented with 5% heat inactivated dialysed foetal calf serum and 40 µCi/ml (^{32}P)-orthophosphate (Amersham, UK) prior to addition of 2 µg/ml PHA (Burroughs Wellcome, UK). Cell lysis and immunoprecipitation were performed as previously described [14]. Briefly, 2×10^7 cells were extracted with 1 ml of lysis buffer (1% Nonidet P40 in 10 m*M* Tris HCl buffer, pH 7.4, containing 0.15 *M* NaCl, 1% BSA, 1 m*M* phenylmethanesulphonyl fluoride, 1 m*M* EDTA and 50 m*M* NaF) for 10 min at 4 °C. After centrifuging for 195000 gmin, lysates were precleared with fixed *Staphylococcus aureus* organisms and rabbit anti-(mouse immunoglobulin). Precleared lysate (1 ml) was precipitated with 5–10 µg of monoclonal antibody UCHT1 [16], covalently coupled to Sepharose 4B beads (Pharmacia Fine Chemicals). Immunoprecipitates were washed sequentially with lysis buffer containing (a) 0.65 *M* NaCl, (b) lysis buffer plus 0.1% SDS, and (c) 0.1% Nonidet P40 in 10 m*M* Tris HCl, pH 7.4, and then analyzed by SDS-PAGE on a 12% gel run under reducing conditions

duced IL-2 production. It is noteworthy that previous studies have shown that phorbol esters can inhibit antigen-mediated proliferative and cytolytic responses [24, 25]. It thus seems likely that there is an intracellular signalling pathway between the phorbol ester target, protein kinase C, and the T3/T cell antigen receptor complex. Exposure to phorbol esters down-regulates the surface expression of T3/Ti [26, 27]. However, the lack of response to anti-T3 antibodies in phorbol ester treated cells is not necessarily due to down-regulation of T3/Ti since, when phorbol esters are removed, T3 levels recover rapidly (within 2–3 h) (unpublished data). The alternative possibility is that exposure to phorbol esters inactivates the transmembrane signalling functions of T3/Ti.

This functional inhibition could be due to the effects of phorbol esters on protein kinase C expression since treatment with phorbol esters greatly reduces cellular levels of

protein kinase C (unpublished data). Nevertheless, protein kinase C is not totally removed by exposure to phorbol esters; thus the possibility exists that the T3/Ti signalling system is inactivated by some other mechanism. One potential mechanism for inactivation is protein kinase C mediated phosphorylation of T3. Phorbol esters induce phosphorylation of the γ subunit of T3 [26]. As well, similar phosphorylation of the T3 γ chain occurs in T cells activated with antigen (data not shown) or a polyclonal activator such as PHA. Thus the data in Fig. 3 show that PHA induces phosphorylation of a M_r 26000 T3 polypeptide that has been identified as the T3 γ chain. It is of interest therefore that T3 phosphorylation is a common feature of T-cell activation with those stimuli that have been shown to down-regulate the surface expression and/or functions of T3/Ti.

D. Discussion

We have used phorbol esters to activate protein kinase C in order to evaluate the role of this kinase in the regulation of T-cell proliferation. We have provided evidence that protein kinase C can deliver positive growth-regulatory signals in the T-cell and initiate IL-2 receptor expression and hence IL-2 responsiveness. As well, stimulation of protein kinase C can induce IL-2 production if a concomitant signal elevating intracellular Ca^{2+} levels is provided. This second stimulus can be generated by a calcium ionophore or by triggering of the T3/Ti complex with anti-T3 antibodies.

A single stimulus with an anti-T3 antibody can initiate IL-2 receptor expression but not IL-2 production. It is not known whether protein kinase C has an intermediate role in this latter signalling system or whether some unidentified pathway is important. A single stimulus with anti-T3 has been shown to induce phosphatidylinositol metabolism and inositol triphosphate release which then generates a Ca^{2+} signal. Nevertheless, elevation of intracellular Ca^{2+} levels is not sufficient to induce IL-2 receptor expression since calcium ionophores were ineffective in this respect. The metabolism of phosphatidylinositol would also generate diacylglycerol which could activate protein kinase C and thus initiate IL-2 receptor expression. However, if T3/Ti triggering delivers the dual signals of Ca^{2+} and protein kinase C activation, there is the discrepancy regarding why this does not result in IL-2 production unless an additional signal such as phorbol ester is also present. One explanation may reside in the predicted differences in the kinetics of protein kinase C activation in response to phorbol esters or endogenous diacylglycerol production. The latter pathway would give a transient activation of protein kinase C whereas phorbol esters would be expected to give prolonged stimulation.

Consequently we would propose that there are two major differences with respect to the signal requirements for induction of the IL-2 and IL-2 receptor genes. Firstly, induction of IL-2 production requires both a Ca^{2+} signal and protein kinase C activation whereas induction of Il-2 receptors requires only protein kinase C activation. Secondly, a transient activation of protein kinase C may be sufficient to induce IL-2 receptor expression whereas a more prolonged stimulation is necessary to ensure Il-2 production. To test this model it will be necessary to establish directly whether a single stimulus of T3/Ti triggering can activate protein kinase C.

Activation of protein kinase C can also deliver a negative signal to T cells, since phorbol esters can down-regulate the surface expression and functions of T3/Ti molecules and inhibit antigen-regulated functions such as cytotoxicity and proliferation. The molecular basis for this regulation may be protein kinase C mediated phosphorylation of the T3 γ chain. There are other examples of receptor functions controlled by phosphorylation/dephosphorylation. For example, desensitisation of α and β adrenergic receptors is associated with their phosphorylation [28, 29]. There is also an interesting parallel in the fibroblasts in which protein kinase C regulates the surface expression and functions of the epidermal growth factor receptor via phosphorylation/dephosphorylation [30–33].

In summary, we would propose a model in which protein kinase C has a dual role in the regulation of T-cell proliferation. Immune

stimulation of T cells via the T3/T cell antigen receptor complex results in activation of protein kinase C, which then functions as a positive signal in the induction of the IL-2/IL-2 receptor genes and may be a critical component of the intracellular mechanisms that regulate IL-2 production and IL-2 receptor expression via T3/Ti. Protein kinase C activation also initiates a negative feedback pathway that terminates the functions of the T3/T-cell antigen receptor complex and may therefore be relevant to the molecular events that determine the transient nature of the T-cell proliferative response. This model is based on the assumption that the biological response to phorbol esters is due solely to the effects of phorbol esters on protein kinase C. Moreover, there is also the assumption that pharmacological activation of protein kinase C with phorbol esters will have effects similar to physiological activation of the kinase. However, it must not be ruled out that phorbol esters can have direct effects on alternative signalling systems, and in this respect it is noteworthy that there are indications of a family of molecules structurally related to protein kinase C [34, 35]. These might also be cellular targets for phorbol esters and may have a role as intracellular signals in the T cell.

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