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T-Cell Proliferation Involving the CD28 Pathway Is Associated with Cyclosporine-Resistant Interleukin 2 Gene Expression

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CD28 is a homodimeric glycoprotein expressed on the surface of a major subset of human T cells that has recently been identified as a member of the immunoglobulin supergene family. The binding of monoclonal antibodies to the CD28 antigen on purified T cells does not result in proliferation; however, previous studies have shown that the combination of CD28 stimulation and protein kinase C activation by phorbol myristate acetate (PMA) results in T-cell proliferation that is independent of both accessory cells and activation of the T-cell receptor-CD3 complex. In the present study, effects of stimulation by anti-CD28 on cell cycle progression and on the interleukin 2 (IL-2) and IL-2 receptor system have been investigated on primary cultures of purified peripheral-blood CD28$^+$ T cells. There was no measurable effect on cell size or on DNA synthesis after stimulation of resting (G0) cells by CD28 alone. After 3 h of activation of T cells by PMA alone, a slight (8%) increase in cell volume occurred that did not progress to DNA synthesis. In contrast, T-cell stimulation by CD28 in combination with PMA resulted in a progressive increase in cell volume in ~100% of cells at 12 to 14 h after stimulation. Northern blot (RNA blot) analysis revealed that CD28 stimulation alone failed to cause expression of the alpha chain of the IL-2 receptor or of IL-2 mRNA, and in accord with previous studies, stimulation by PMA alone resulted in the accumulation of IL-2 receptor transcripts but no detectable IL-2 mRNA. In contrast, T-cell stimulation by the combination of CD28 and PMA resulted in the appearance of IL-2 transcripts and enhanced expression of IL-2 receptor mRNA. Functional studies revealed that the proliferation induced by CD28 and PMA stimulation was entirely resistant to cyclosporine, in contrast to T-cell activation induced by the CD3-T-cell receptor complex. Cyclosporine was found not to affect the accumulation of IL-2 mRNA after CD28 plus PMA stimulation, although there was no detectable IL-2 mRNA after stimulation by CD3 in the presence of the drug. Furthermore, stimulation by CD28 in combination with immobilized CD3 antibodies caused a striking enhancement of IL-2 mRNA expression that was, in part, resistant to the effects of cyclosporine. These studies indicate that the CD28 molecule synergizes with protein kinase C activation to induce IL-2 gene expression and demonstrate that stimulation by the CD28 pathway can cause vigorous T-cell proliferation even in the presence of cyclosporine and that cyclosporine does not prevent transcription of 16-2 mRNA, as has been suggested previously. Moreover, these findings suggest that a potential role for the CD28 molecule in vivo may be to augment IL-2 production after stimulation of the CD3-T-cell receptor molecular complex and thereby to amplify an antigen-specific immune response. Finally, these results provide further evidence that the CD28 molecule triggers T-cell proliferation in a manner that differs biochemically from CD3-T-cell receptor-induced proliferation.

The binding of antigen or monoclonal antibodies to the T-cell receptor or the closely associated cluster designation 3 (CD3; see reference 51 for description of CD nomenclature) molecular complex initiates a cascade of biochemical events consisting of phosphatidylinositol hydrolysis, increased intracellular free calcium concentration, membrane translocation of protein kinase C, and increased cytoplasmic cyclic AMP (cAMP) concentration (15, 25, 27, 34, 36, 49, 59). In the presence of surface receptor cross-linking (33, 34, 64), these early signals are followed by activation of the genes for the interleukin 2 receptor (IL-2R) and interleukin 2 (IL-2) and by subsequent T-cell proliferation (4). Similarly, binding of the CD2 ligand termed LFA-3 (48) or of monoclonal antibody (MAb) to the CD2 sheep E rosette receptor results in the activation of T cells and large granular lymphocytes (27, 40). The biochemical events that accompany activation by CD2 appear to be identical to activation via the CD3-T-cell receptor pathway (27, 44).

CD28 is a cell surface antigen first defined by MAb 9.3 (21). CD28, previously termed Tp44, is homodimeric glycoprotein that was recently cloned and shown to have substantial homology to immunoglobulin genes (1). The CD28 molecule is expressed on the surface of most mature T cells; it is found on ~95% of CD4$^+$ and on ~50% of CD8$^+$ T cells (9). This subset of T lymphocytes has distinct functional abilities, such as the ability to provide helper function to B cells and to kill cells infected with virus, yet does not contain the population of T cells that display suppressor function (9, 38).

The first indication that CD28 might function as an alternative T-cell activation pathway was the observation that MAb 9.3 caused marked enhancement of T-cell proliferative responses to phytohemagglutinin (PHA) and allogeneic cells (17). It was subsequently found that MAb 9.3 also caused marked augmentation of T-cell proliferation induced by CD3 antibodies (39, 60) and that the combination of MAb 9.3 and phorbol myristate acetate (PMA) could induce vigorous proliferation of T cells that was independent of accessory cells (23, 42). T-cell proliferation induced by CD28 appears

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to be distinct from that induced by the CD3-T-cell receptor because T cells with the CD3 molecular complex removed from the cell surface still respond to CD28 stimulation (23, 42), whereas they do not after CD2 stimulation (40). In contrast to stimulation by CD3, stimulation of T cells by CD28 leads to an increased concentration of cytoplasmic cyclic GMP (cGMP) (36) and, in the absence of cross-linking, does not cause increased intracellular calcium concentration (33, 36) or translocation of protein kinase C (36, 60). Agents that increase the cytoplasmic cAMP concentration inhibit IL-2 production subsequent to stimulation of cells by immobilized CD3 (7); however, the addition of CD28 MAb to cells stimulated by CD3 conferred resistance to the effects of cAMP (36). Furthermore, the CD3 signal causes phosphorylation of the $pp60^{c\text{-}src}$ tyrosine kinase, whereas the CD28 signal does not (32). Thus, accumulating evidence indicates that CD28 initiates or regulates T-cell proliferation in a major subset of T cells in a manner that differs biochemically from CD3-induced T-cell proliferation. In the present report we have studied the mechanism of T-cell activation by CD28. The CD28 signal synergizes early in the cell cycle with phorbol esters, resulting in IL-2 gene activation, although neither the CD28 signal nor the signal provided by phorbol ester alone was sufficient for IL-2 induction. In contrast to T-cell proliferation induced by the CD3 pathway, proliferation induced by the CD28 pathway was found to be entirely resistant to cyclosporine, and furthermore, it was demonstrated that CD28-induced IL-2 gene expression is resistant to cyclosporine.

MATERIALS AND METHODS

MAbs. CD28 MAb 9.3 was produced as described previously (35). The antibody was purified on protein A-Sepharose, dialyzed against phosphate-buffered saline, filtered (0.22-μm-pore-size sterile filter), and cleared of aggregates by centrifugation (100,000 × *g* for 45 min) prior to use in functional assays. CD3 MAb G19-4 was produced and purified as described previously (36). In functional assays, MAb 9.3 was added at the previously determined optimal concentration of 100 ng/ml to cells at 0.5×10^6/ml unless otherwise stated (23), and MAb G19-4 was used after first being adsorbed to the surface of plastic tissue culture plates as described previously (33). This was done because of the requirement for cross-linking to achieve CD3-mediated proliferation of purified T cells (16, 33, 64), while cells stimulated with CD28 plus phorbol ester do not require additional cross-linking (23). CD7 MAb G3-7 (33) was used as a control antibody that binds to all $CD28^+$ cells.

Cells. Buffy coats were obtained by leukopheresis of healthy donors aged 21 to 31 years. Peripheral blood lymphocytes (PBL) ($\sim 2.5 \times 10^9$) were isolated from the buffy coat by Lymphocyte Separation Medium (Litton Bionetics, Kensington, Md.) density gradient centrifugation. Monocytes were depleted from the PBL by plastic absorption for 2 h at 37°C. The $CD28^+$ subset of T cells was then isolated from the plastic-nonadherent cells by negative selection with immunoabsorption. This strategy takes advantage of the reciprocal and nonoverlapping distribution of the CD11 and CD28 surface antigens on T lymphocytes (67). Cells were suspended at 20×10^6/ml in RPMI 1640 medium containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4; GIBCO Laboratories, Grand Island, N.Y.), 5 mM EDTA, and 5% heat-inactivated human AB serum (Pel-Freez, Brown Deer, Wis.). The cells were then incubated at 4°C on a rotator with saturating amounts of MAbs 60.1 (CD11), 1F5 (CD20), FC-2 (CD16), and 20.3 (CD14) for 20 min. Thus, this mixture of antibodies coated all B cells, monocytes, large granular lymphocytes, and $CD28^-$ T cells with mouse immunoglobulin. The cells were washed three times to remove unbound antibody and then incubated with goat anti-mouse immunoglobulin-coated magnetic particles (Advanced Magnetics Institute, Cambridge, Mass.) at a ratio of 50 particles per cell. Antibody-coated cells that were bound to beads were then removed by magnetic separation, and typically $\sim 700 \times 10^6$ $CD28^+$ T cells were recovered. Cell purification was routinely monitored by flow cytometry and histochemistry. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated MAb T11 (CD2; Coulter Electronics, Inc., Hialeah, Fla.) and with FITC-conjugated MAb 9.3 (CD28) and were >99% and >98% positive compared with a nonbinding, isotype-matched, FITC-labeled control antibody. Monocytes, B cells, and large granular lymphocytes were not detectable by immunofluorescence analysis. Residual monocytes were quantitated by staining for nonspecific esterase and constituted <0.1% of all cell populations used in this study. Viability was >99% as measured by trypan blue exclusion.

Proliferation assays. Cells were cultured in quadruplicate samples in flat-bottomed 96-well microtiter plates at 5×10^4 cells per well in Iscove medium containing 5% heat-inactivated fetal calf serum (lot 1114594; Hyclone, Logan, Utah). Cell proliferation was measured in a liquid scintillation counter after pulsing cells for the last 8 h of 3-day cultures with 1 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per well.

Measurement of cell volume. Cells were sized on a linear scale with a Coulter Counter model ZM (Coulter Electronics) equipped with a cylindrical 70-μm aperture and a Channelyzer model C-1000 (Coulter Electronics) interfaced to an Apple IIE computer. Cells were suspended in phosphate-buffered saline, and calibration was performed by using latex beads 5.0 and 9.70 μm in diameter. A shape factor of 1.38 was used (47). The results of each analysis were stored as a histogram containing 100 data points, although for clarity, histograms of cell volume are presented as a smooth curve containing symbols used for identification purposes only.

Northern blot (RNA blot) analysis. $CD28^+$ T cells were cultured at 2×10^6/ml in complete medium. The cells were harvested by centrifugation, and total cell RNA was extracted with guanidinium isothiocyanate (6). The samples were equalized for rRNA, and the equalization was confirmed by ethidium bromide staining of equal amounts of the RNA samples separated on a nondenaturing 1% agarose gel as described previously (56). These equalized RNA samples (5 to 10 μg) were separated on 1% agarose–formaldehyde gels and transferred to nitrocellulose. Membranes were baked under vacuum for 2 h and then prehybridized at 42°C in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution, 25 mM sodium phosphate (pH 6.5), and 250 μg of *Torula* RNA per ml. Hybridizations were carried out for 16 to 20 h under identical conditions except for the addition of 10% dextran sulfate and 10^6 cpm of the DNA probe per ml. DNA probes were labeled by nick translation to a specific activity of 3×10^8 to 9×10^8 cpm/μg. Membranes were washed briefly at room temperature following hybridization in 2× SSC–0.1% sodium dodecyl sulfate and then twice for 30 min each at 56°C in 0.1× SSC–0.1% sodium dodecyl sulfate. The membranes were then air dried and exposed to X-ray film (Kodak XAR-2) for 4 to 36 h at −70°C with

intensifying screens. Band intensities were compared by densitometry as described previously (56).

DNA probes. The ^{32}P-labeled probes used in these experiments were all the result of nick translation of gene-specific inserts (50 to 100 ng) isolated from low-melting-point agarose gels following digestion of the plasmid in which they were propagated with appropriate restriction endonucleases. The IL-2-specific probe is a 1.0-kilobase *Pst*I cDNA insert derived from the pTCGF5 plasmid, obtained from S. Aria (8). The IL-2 alpha-chain receptor probe is a 1.9-kilobase *Eco*RI-*Bam*HI fragment derived from pIL2R3, obtained from W. Greene (37). The human leukocyte antigen (HLA) B7 probe was a 1.4-kilobase *Pst*I fragment isolated from pHLA-B7 (53).

Reagents. Cyclosporine (Sandoz, Hanover, N.J.) was dissolved in ethanol-Tween 80 exactly as described by Wiesinger et al. (63) and stored as a 20-mg/ml stock solution. 4-α-Phorbol-12,13-didecanoate, 4-α-phorbol-12-myristate-13-acetate, 4-β-12-myristate-13-acetate (PMA), and phorbol-12,13-dibutyrate were from LC Services Corp., Woburn, Mass. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was prepared fresh as a 1-mg/ml stock solution. PHA was obtained from Sigma. Human recombinant IL-2 was provided by Genzyme, Boston, Mass.

RESULTS

Effects of CD28 stimulation on DNA synthesis. Protein kinase C is thought to account for all or most of the biological events that occur after treatment of cells with PMA (45). In previous studies that used primary cultures of unfractionated PBL, PMA was reported to be a T-cell mitogen with a potency approximately one-third that of the lectin PHA (58). After treatment of purified CD28$^+$ cells with PMA only, thymidine incorporation was only slightly above the baseline (Table 1), indicating that PMA is a comitogen for T cells, and that "accessory" cells (monocytes) are required for PMA to cause T-cell proliferation (10). When cells were stimulated with MAb 9.3 only, proliferation on day 3 of culture was not above that of cells cultured in medium. In contrast, when cells were cultured with the combination of MAb 9.3 and PMA, large amounts of thymidine incorporation were observed. Similar results were obtained by Hara et al. with unfractionated, rigorously purified T cells (23). The synergy observed with MAb 9.3 with PMA was specific, as the binding of CD7 (Table 1), CD5, CD6, and CD45 (not shown) with MAbs to cells in the presence of PMA did not increase thymidine incorporation above that with PMA alone. PHA, like PMA, is a T-cell mitogen that is dependent on accessory cells (66). We found that optimal doses of PHA, in the presence or absence of recombinant IL-2, were unable to cause significant thymidine incorporation, although there was vigorous proliferation when cells were stimulated with the combination of PHA plus MAb 9.3, indicating that PHA, like PMA, provides a signal that is comitogenic with CD28. The small amounts of thymidine incorporation observed when purified CD28$^+$ T cells were stimulated by PMA alone and by PHA alone may be due to the small (<0.1%) residual population of accessory cells. Alternatively, it may indicate the presence of cells partially activated in vivo or during the isolation procedure. When cells were cultured with MAb 9.3, PMA, or MAb 9.3 plus PMA and thymidine incorporation was measured daily for 5 days, we found that no proliferation was induced by either MAb 9.3 or PMA above that shown for day 3 and that 9.3- plus PMA-induced proliferation peaked on days 3 and 4 of culture (data not shown).

TABLE 1. Effects of CD28 stimulation on T-cell proliferation[a]

Stimulation	Mean [^{3}H]thymidine incorporation (cpm) ± 1 SD
Medium	55 ± 12
CD28 MAb 9.3	84 ± 44
CD7 MAb G3-7	58 ± 21
PMA	407 ± 251
PMA + MAb 9.3	71,600 ± 690
PMA + MAb G3-7	927 ± 686
PHA	620 ± 367
PHA + IL-2	1,600 ± 692
PHA + MAb 9.3	78,500 ± 12,000
PHA + MAb G3-7	283 ± 100

[a] PBL CD28-positive T cells were purified and cultured as described in the text, and the arithmetic mean thymidine incorporation was measured on day 3. Conditions were MAb 9.3 at 100 ng/ml, MAb G3-7 at 100 ng/ml, PMA at 1 ng/ml, PHA at 10 μg/ml, and recombinant IL-2 at 20 U/ml.

Effects of CD28 stimulation on cell size. To study further the synergistic effects of CD28 and protein kinase C activation, we evaluated the effects of these signals on cell size. Changes in cell size have been shown to correlate with cell cycle activation and in particular with G0 to G1 transition (11, 41, 57). Shown in Fig. 1 are histograms of cell volume at 3, 14, 18, and 24 h of culture. When cells were cultured in various combinations of medium, PMA, CD28 MAb 9.3 and CD7 MAb G3-7, a small but reproducible 8% volume enlargement was found in cells cultured for 3 h in PMA alone (Fig. 1, upper left panel). At 3 h, there was no measurable effect of stimulation by MAb 9.3 itself, as the median volume for cells cultured in medium or MAb 9.3 only remained identical at 210 fl, and the volume of cells stimulated with 9.3 plus PMA was equivalent to that with PMA alone. After 14 h of culture, however, the median size of cells stimulated with MAb 9.3 plus PMA had increased to 318 fl, exceeding that induced by PMA alone at 275 fl, while the volume of cells stimulated with CD7 control antibody and CD28 MAb remained equivalent to that of cells cultured in medium only. At 18 and 24 h after stimulation, cells cultured in 9.3 plus PMA continued to enlarge and had a median volume of 409 fl at 24 h. In contrast, cells stimulated with PMA with or without CD7 MAb G3-7 did not continue to enlarge, and furthermore, cells stimulated with the control CD7 antibody plus PMA did not enlarge more than cells cultured in PMA only. The sizing histogram for cells stimulated with 9.3 plus PMA was unimodal and shifted to the right in comparison with that for PMA-stimulated cells, suggesting that all cells respond to the addition of the CD28 signal. No effect of CD28 MAb 9.3 itself was observed on cell size at 24 h (Fig. 1, lower right) or at 36 h (not shown). The above results indicate that stimulation by CD28 of purified CD28$^+$ T cells does not increase cell size and presumably does not mediate a G0 to G1 transition. The CD28 signal synergizes with PMA to cause cell enlargement and cell cycle progression that is first evident at 12 to 14 h after stimulation.

Effects of CD28 stimulation on IL-2 and IL-2R gene expression. Northern blot analysis was used to characterize further the CD28 and PMA signals. Purified CD28$^+$ T cells were cultured with CD28 MAb 9.3, PMA, or 9.3 plus PMA for 1, 6, 12, or 36 h, and total cellular RNA was prepared (Fig. 2). Since rRNA increases in direct proportion to cell volume as cells progress through the cell cycle, each sample was equalized for the level of rRNA (Fig. 2, top panel) to avoid the measurement of mRNA variations attributable only to increases in cell volume. Northern blots were hybridized sequentially with probes specific for the alpha chain of the

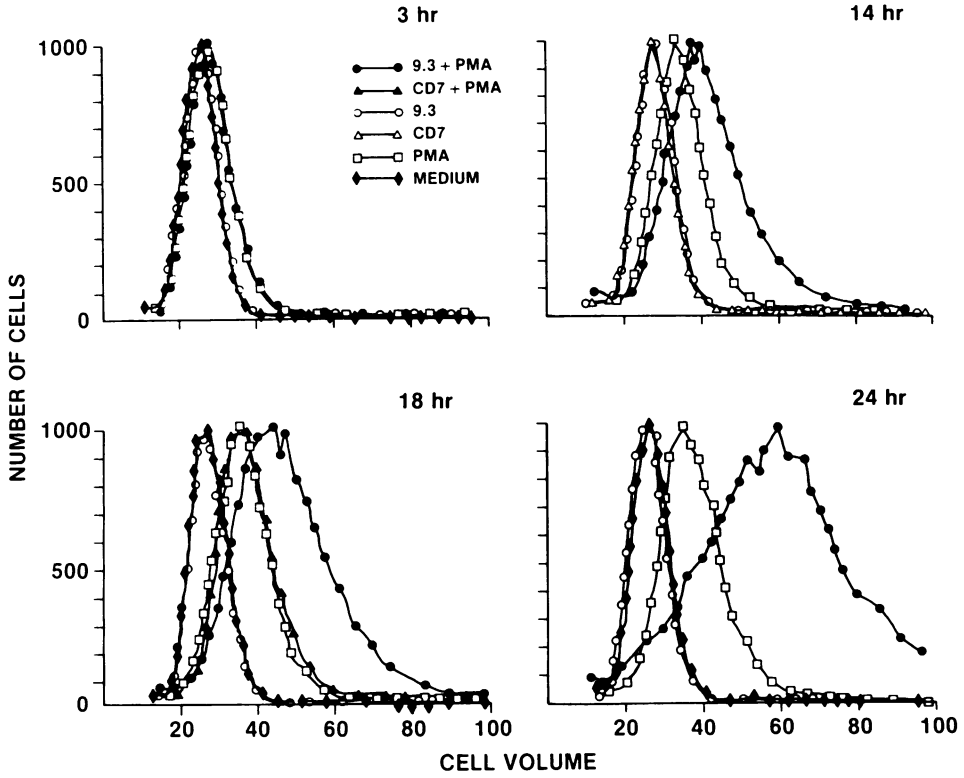

FIG. 1. Effects of CD28 MAb 9.3 and PMA on cell size. CD28$^+$ T cells were cultured with medium, PMA (1 ng/ml), CD28 MAb 9.3 (100 ng/ml), or control CD7 MAb G3-5, and cell volume was determined (57). Histograms of cell volume (channel number) versus number of cells are shown from an experiment at the indicated time points; for purposes of clarity, all conditions are not depicted at each time point. Debris and dead cells were excluded by measuring particles larger than 75 fl. Latex beads having volumes of 65 and 491 fl had median channel values of 5 and 81, respectively. Data are representative of three independent experiments.

IL-2 receptor, IL-2, and a class I HLA gene was used to serve as an mRNA probe for a constitutively expressed gene. Cells stimulated with MAb 9.3 only did not express detectable mRNA for IL-2R or IL-2 at any time point.

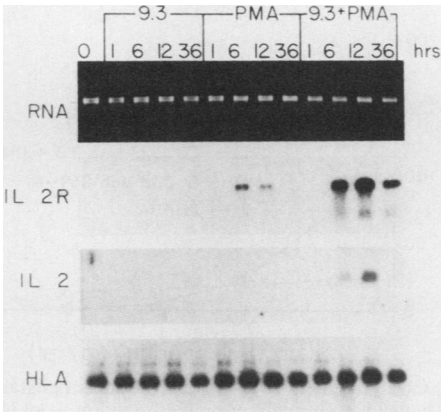

FIG. 2. Time course of IL-2 and IL-2R gene expression. CD28$^+$ T cells were stimulated with CD28 MAb 9.3, PMA, or MAb 9.3 plus PMA as described in the text and harvested at the indicated times of culture. RNA was isolated and equalized for rRNA as described (6, 56). The equalization was confirmed by ethidium bromide staining of 10% portions of each RNA sample separated on a nondenaturing 1% agarose gel (upper panel). Northern blots were prepared, and the filters were hybridized sequentially with cDNA probes specific for the alpha chain of the IL-2 receptor, IL-2, and HLA (lower panels).

mRNA specific for IL-2R was expressed at 6 and 12 h after treatment with PMA, although no IL-2 transcripts could be detected. In contrast, when cells were stimulated with 9.3 plus PMA, mRNA specific for IL-2 was detectable at 6 and 12 h of culture. In addition, higher levels of IL-2R mRNA were found after 9.3 plus PMA stimulation than after stimulation by PMA alone. The level of class I HLA gene expression did not show any significant variation with any of the conditions tested. The results shown in Fig. 2 are representative of four independent experiments.

The above result confirmed that activation of protein kinase C alone was not sufficient to cause IL-2 mRNA expression (12, 62, 63). We next performed a series of experiments to ask whether CD28 stimulation itself could affect IL-2 expression. CD28$^+$ T cells were cultured with MAb 9.3 at 10-fold (1 μg/ml) or 100-fold (10 μg/ml) higher concentrations than those used for the experiments shown in Fig. 1 and 2, and the cells were harvested after 12 h of culture (Fig. 3). No IL-2 mRNA was detected in cells stimulated with high doses of MAb 9.3 only, although the culture containing MAb 9.3 at 0.1 μg/ml plus PMA had easily detectable IL-2 expression. Protein kinase C activation was required for collaboration with the CD28 signal, as cells cultured with the biologically inactive phorbol ester 4-α-phorbol-12,13-didecanoate and MAb 9.3 did not have IL-2 mRNA. Transcripts hybridizing with the HLA class I gene were detectable at comparable levels at all time points sampled, confirming equalization of the blot and excluding mRNA degradation as an explanation for the failure to detect IL-2 mRNA in the cultures stimulated with MAb 9.3 only.

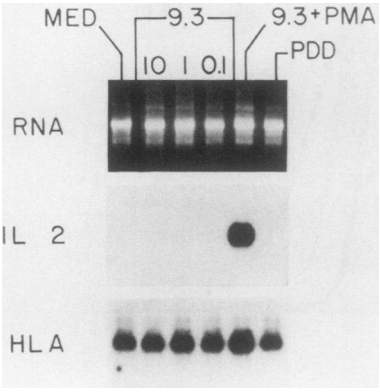

FIG. 3. Stimulation by CD28 MAb 9.3 is not sufficient to induce IL-2 gene expression in purified T cells. $CD28^+$ T cells were cultured with medium (MED), MAb 9.3 at 10, 1, or 0.1 μg/ml, or MAb 9.3 (0.1 μg/ml) plus PMA (1 ng/ml) or 4-α-phorbol-12,13-didecanoate (PDD) (1 ng/ml). The cells were harvested after 12 h of culture, and RNA was isolated and equalized (upper panel) as described in the text. Northern blots were hybridized sequentially with IL-2- and HLA-specific cDNA probes.

Given the result that the CD28 signal synergized with protein kinase C activation to result in IL-2 gene expression, it was possible that one signal acted at a transcriptional level to induce mRNA transcription and that the other might act at a posttranscriptional level to enhance the stability of IL-2 transcripts. Previous reports have shown that the mRNA levels of another lymphokine, granulocyte-macrophage colony-stimulating factor, are in part regulated by mRNA degradation and that an RNA degradation pathway is controlled by protein kinase C (50). Furthermore, many rapidly degraded mRNAs can be stabilized by transient inhibition of protein synthesis, suggesting that a labile protein regulates mRNA stability. To examine whether the accumulation of IL-2 mRNA following stimulation by CD28 only or by PMA only was prevented by rapid degradation, cells were stimulated for 6 or 24 h in the presence or absence of cycloheximide, an inhibitor of protein synthesis that was added for the last 2 h of culture (Fig. 4). Even in the absence of protein synthesis, the CD28 signal was not able to induce detectable amounts of IL-2 mRNA. Similarly, PMA itself was not able to induce IL-2 expression in the presence of cycloheximide, indicating that interruption of the translation of a constitutive protein with a short half-life is not sufficient to permit accumulation of IL-2 transcripts. In contrast, when cells were stimulated by CD28 plus PMA, there was a 5- to 10-fold enhancement of IL-2 mRNA levels following cycloheximide treatment at both time points sampled, suggesting, by analogy with other lymphokines (5, 50), that the level of IL-2 mRNA is regulated in part by modulation of mRNA stability.

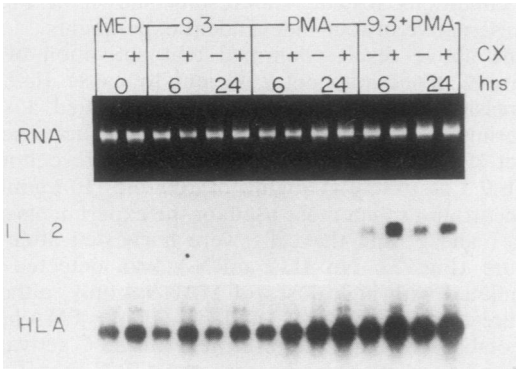

FIG. 4. MAb 9.3 is unable to induce IL-2 gene expression in the absence of protein synthesis. $CD28^+$ T cells were cultured in duplicate with medium (MED), MAb 9.3, PMA, or 9.3 plus PMA for the intervals shown (in hours). During the last 2 h of culture, the protein synthesis inhibitor cycloheximide (CX) (10 μg/ml) was added as indicated. Total cellular RNA was isolated and equalized for rRNA (upper panel). Northern blots were hybridized sequentially with IL-2- and HLA class I-specific cDNA probes.

CD28-induced T-cell proliferation is resistant to the effects of cyclosporine. Together, the above results indicate that neither CD28 nor PMA stimulation alone was able to induce IL-2 mRNA and that the two signals together were sufficient for IL-2 production and T-cell proliferation. Cyclosporine is a potent immunosuppressant that is relatively specific for T cells (52, 64). We investigated the effects of cyclosporine on CD28-induced activation, as there is evidence that a mechanism of action of cyclosporine is to prevent IL-2 gene expression but not IL-2R expression (14, 18, 30, 31). T cells enriched by nylon wool filtration were cultured with MAb 9.3 plus PMA, CD3 MAb G19-4, or PMA only in the presence of increasing amounts of cyclosporine (Fig. 5). We confirmed previous findings that T-cell proliferation induced by the CD3-T-cell receptor complex is sensitive to cyclosporine, with a 50% effective dose of ~25 ng/ml (29, 31, 64). In contrast, proliferation induced by the CD28 pathway was entirely resistant to cyclosporine, even at concentrations of cyclosporine as high as 3 μg/ml. This result was observed on eight consecutive experiments with PBL from different donors, indicating that the resistance to cyclosporine of T cells stimulated in this manner is reproducible and does not reflect variations among normal individuals. The concentration of cyclosporine used in these experiments exceeded those that are achievable in vivo. The cells used for the experiment in Fig. 5 were enriched for T cells but cannot be considered pure, as ~1% monocytes remained. Enriched rather than purified T cells were used in Fig. 5 to demonstrate that PMA can be a T-cell mitogen under these conditions and that this effect was inhibited ~50% by cyclo-

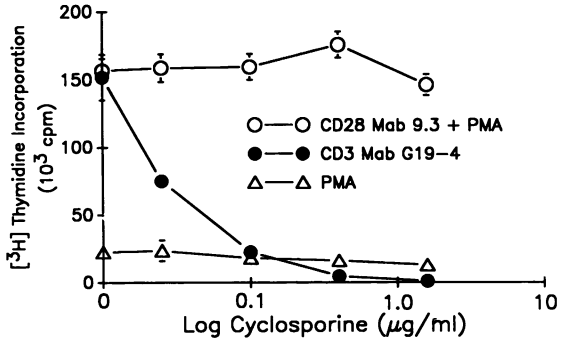

FIG. 5. CD28-induced T-cell proliferation is resistant to cyclosporine. T cells (5×10^4/well) enriched by nylon wool filtration (26) were cultured with MAb 9.3 (100 ng/ml) plus PMA (1 ng/ml), plastic-adherent MAb G19-4, or PMA (100 ng/ml), and fourfold titrations of cyclosporine from 25 ng/ml to 1.6 μg/ml; the cyclosporine diluent did not affect proliferation (data not shown). Thymidine incorporation was measured on day 3 of culture and the arithmetic mean ± 1 standard deviation is depicted where the error bar exceeds the size of the symbol. Proliferation of cells cultured in medium alone was 185 ± 40 cpm. Data are representative of eight independent experiments.

TABLE 2. Effects of cyclosporine on CD3-mediated proliferation of T cells[a]

Stimulus	Mean [^{3}H]thymidine incorporation (kcpm) ± 1 SD at cyclosporine concn (μg/ml):					% Inhibition
	0	0.2	0.4	0.8	1.2	
CD3 MAb G19-4	77 ± 26	61 ± 6.8	52 ± 4.4	10 ± 3.4	8.2 ± 1.2	90
CD3 + CD28 MAb 9.3	123 ± 18	86 ± 2.3	63 ± 4.4	44 ± 6.4	43 ± 5.2	65
CD3 + PMA	145 ± 12	132 ± 2.8	123 ± 6.4	55 ± 3.6	56 ± 6.4	62
CD28 MAb 9.3 + PMA	111 ± 12	97 ± 5.6	107 ± 12	99 ± 14	112 ± 2.4	<0

[a] CD28-positive T cells were cultured, and thymidine incorporation was determined on day 3 of culture. The conditions tested were: CD3 antibody adsorbed onto plastic; MAb 9.3, 100 ng/ml; and PMA, 1 ng/ml. The percent inhibition of proliferation was calculated between cells cultured in medium only or in cyclosporine at 1.2 μg/ml. Cells cultured in the absence of cyclosporine were given cyclosporine diluent. Thymidine incorporation of cells cultured in medium, PMA, or MAb 9.3 only was <150 cpm.

sporine. Previous studies of tumor cell lines stimulated with phorbol diesters have shown partial resistance to the effects of cyclosporine (31). When purified CD28$^+$ T cells were cultured with cyclosporine, proliferation induced by immobilized CD3 was inhibited and CD28- plus PMA-stimulated proliferation was again found to be entirely resistant to cyclosporine (Table 2), indicating that the CD28 pathway is also resistant to the effects of the drug under conditions in which the signal provided by PMA is not mitogenic. We then used previous observations that CD28 MAb 9.3 or PMA can augment proliferation induced by CD3 (22, 35) to ask whether the resistance to the effects of cyclosporine was conferred by PMA or by CD28. In the context of CD3-T-cell receptor stimulation, we found that neither signal was able to confer complete resistance to cyclosporine and that the addition of either CD28 or PMA to CD3-stimulated cultures resulted in less sensitivity to the drug than to stimulation by immobilized CD3 alone (Table 2).

Cyclosporine does not inhibit IL-2 mRNA expression. Given the result that proliferation induced by stimulation with CD28 plus PMA was resistant to cyclosporine and that the combination of signals was able to induce IL-2 gene expression, Northern blots were prepared to determine the effects of cyclosporine on IL-2 mRNA expression. CD28$^+$ T cells were stimulated with either CD28 MAb 9.3 plus PMA or CD3 with and without MAb 9.3 (Fig. 6 and 7). There was full expression of IL-2 mRNA in cells cultured for 12 h with 9.3 plus PMA in the presence of cyclosporine at 1 μg/ml. The time course of IL-2 mRNA expression was identical in cells treated with cyclosporine and in those cultured in the absence of cyclosporine (Fig. 2 and 6). Cyclosporine was tested at 0.5, 1.0, and 1.5 μg/ml and found to have no meaningful inhibitory effect on IL-2 mRNA expression on cells stimulated by CD28 plus PMA even at the highest dose of the drug tested.

Previous work has shown that the addition of CD28 stimulation to cells stimulated optimally by immobilized CD3 does not increase proliferation (16), and we have found that immobilized CD3 causes proliferation that is quantitatively similar to that of cells stimulated by CD28 plus PMA (Fig. 5 and Table 2). Cells were also cultured with immobilized CD3 with or without MAb 9.3 in the presence or absence of cyclosporine, and Northern blots were prepared (Fig. 7). After 12 h of culture, surprisingly little IL-2 mRNA was induced in CD3-stimulated cells compared with CD28- plus PMA-stimulated cells (Fig. 6); in the case of CD28- plus PMA-stimulated cells, the film was exposed for 4 h, and for the cells stimulated by immobilized CD3, the film was exposed for 16 h. The RNA from Fig. 6 and 7 was derived from the same experiment, and equal amounts of rRNA were analyzed for each condition and probed simultaneously. While cells stimulated with immobilized CD3 only did express IL-2 mRNA, the addition of soluble CD28 MAb 9.3 to cells stimulated by immobilized CD3 caused a marked enhancement of IL-2 production. In cells treated with CD3

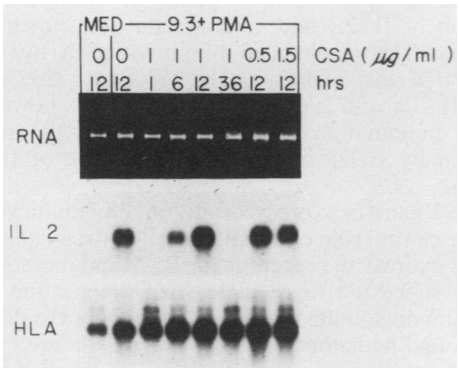

FIG. 6. Failure of cyclosporine (CSA) to inhibit IL-2 mRNA production induced by MAb 9.3 plus PMA. CD28$^+$ T cells were cultured with medium (MED) for 12 h, MAb 9.3 plus PMA for 12 h, MAb 9.3 plus PMA plus cyclosporine (1 μg/ml) for 1, 6, 12, and 36 h, and MAb 9.3 plus PMA plus cyclosporine (0.5 or 1.5 μg/ml) for 12 h. After the cells were harvested at the indicated times, RNA was extracted and equalized for rRNA (upper panel). Northern blots were hybridized sequentially with IL-2- and HLA-specific cDNA probes, and the film was exposed for 4 h.

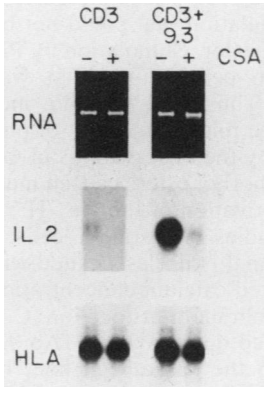

FIG. 7. CD28 augments CD3-induced IL-2 expression in the presence or absence of cyclosporine (CSA). Purified CD28$^+$ T cells were cultured with plastic-adherent CD3 antibody G19-4 with or without MAb 9.3 at 100 ng/ml for 12 h in the absence or presence of cyclosporine (1 μg/ml). The cells were harvested, and the RNA was extracted and equalized for rRNA (upper panel). Blots were hybridized sequentially with IL-2- and HLA-specific cDNA probes. Exposure time for the film from the CD3 culture was 16 h; the film from blots of the CD3 plus 9.3 culture was exposed for 4 h.

alone, IL-2 mRNA expression was completely abolished in the presence of cyclosporine (Fig. 7); no IL-2 could be detected even after exposure of the film for 11 days. In contrast, the IL-2 production of cells cultured with immobilized CD3 plus CD28 MAb 9.3 in the presence of cyclosporine was only partially inhibited, and the cells stimulated with CD28 plus CD3 in the presence of cyclosporine had still greater amounts of IL-2 mRNA than cells cultured in the absence of the drug and stimulated by immobilized CD3 only. Class I HLA mRNA expression was not affected by cyclosporine.

DISCUSSION

We have shown that two signals, one delivered by CD28 and the other by phorbol diesters, are sufficient to cause both IL-2 gene activation and T-cell proliferation. This system provides a useful model to dissect T-cell activation: one signal is provided by phorbol esters and presumably consists of protein kinase C activation, while the nature of the second signal that is furnished by the binding of antibody to CD28 remains undefined. The predicted sequence homology from the cDNA clone of CD28 with immunoglobulin genes (1) suggests that the molecule is a receptor for an unidentified ligand. Uncovering the role of the CD28 molecule in the integrated immune response will await identification of the ligand; however, one can speculate that the pathway exists in vivo, with the CD28 ligand providing one signal, and that the signal provided by PMA in vitro is derived in vivo from cross-linking the CD3 complex (34, 65) or perhaps from cross-linking the CD28 molecule itself.

The mechanism of the signal delivered by the CD28 molecule remains undefined. The CD28 pathway is unique in that it activates a subset of T cells. To study the pathway, it was necessary to use purified $CD28^+$ cells and thereby exclude the possibility that the synergistic effects of the two signals were the result of interactions between different subpopulations of T cells. Binding of MAb 9.3 to CD28 did not cause increases in cell volume, indicating that the cells remained in the G0 phase of the cell cycle. The present results do indicate that the CD28 signal has effects early in the cell cycle, as the addition of phorbol ester to the CD28 signal resulted in a progressive increase in cell volume at 12 to 14 h after stimulation that could not be attributed to the effects of phorbol ester. Stimulation by PMA alone caused a slight increase in cell volume that was independent of accessory cells. Thus, cell swelling induced by PMA is independent of the mitogenic effect of phorbol esters and is likely explained by the observations of Grinstein et al. (19), who found that phorbol esters caused murine thymocytes to enlarge due to activation of the Na^+/H^+ antiport.

In previous studies of resting T cells, it has been found that the CD28 signal is not associated with increases in the cytoplasmic ionized calcium concentration (36) or with protein kinase C membrane translocation (C. H. June and K. E. Meier, unpublished data). When MAb 9.3 is added at high concentrations to the Jurkat cell line, however, small increases in calcium concentration occur that are not accompanied by measurable translocation of protein kinase C (61). This discrepancy probably stems from differences between primary cultures of resting (G0) T cells and leukemic cell lines, as well as the presence of aggregates of antibody that facilitate cross-linking of the CD28 molecule, because we found that cross-linking of MAb 9.3 with a second-step anti-immunoglobulin caused increased T cell cytoplasmic calcium concentration, while soluble MAb 9.3 did not affect the ionized calcium concentration (33). In previous studies we found that CD28 could augment IL-2 secretion in CD3-stimulated cultures of PBL and that the effect was independent of the Fc region of antibody 9.3 but did require bivalent binding, as Fab'_2 fragments were as effective as intact antibody, while Fab fragments were inactive (39). Although we have found that additional cross-linking of the CD28 antigen with antibody 9.3 and a second-step anti-immunoglobulin increases CD28 functional activity and causes calcium mobilization (31), in the present study, intact soluble MAb 9.3 was used under conditions designed to cause minimal cross-linking. Therefore, the present study has characterized the potent signal delivered by CD28 in the absence of detectable alteration of cytoplasmic calcium concentration and that is comitogenic for resting T cells with PHA, PMA, and solid-phase anti-CD3 MAbs.

At least two distinct signals are required for the activation of resting T cells (18, 62, 63, 65). CD28 stimulation does elevate cGMP concentrations (36), a process often associated with cell cycle progression in diverse cell types (20, 59). Thus, the present results suggest a potential mechanism for IL-2 gene activation, with one signal derived from CD28 stimulation resulting in increased cGMP concentration, and the other signal from phorbol diesters presumably resulting from activation of protein kinase C. Elucidation of the molecular events triggered by these "second messengers" that cause IL-2 gene expression will await clarification of the substrates for protein kinase C and cGMP-dependent kinase.

The addition of cycloheximide to CD28-stimulated cells caused increased levels of IL-2 mRNA and, by analogy with the data of Shaw et al. (50) and Caput et al. (5), support the hypothesis that an mRNA degradation pathway is involved in the regulation of mRNA levels of genes that have AU-rich sequences in the 3′ noncoding regions. Our results with CD28 stimulation are similar to those of a previous study by Effrat et al. (13) that suggested that cycloheximide treatment prevented downregulation of IL-2 mRNA in PHA-stimulated lymphocytes. Previous studies have shown that stimulation by protein kinase C alone is sufficient to induce expression of the alpha chain of the IL-2R (12, 18). The present study demonstrates that the CD28 signal did not cause activation of IL-2R, IL-2, c-*myc* (data not shown), or c-*myb* (data not shown) gene transcription. In contrast, the combination of phorbol ester and CD28 stimulation caused enhanced expression of IL-2R and c-*myc* (data not shown) mRNA transcripts. The mechanism of potentiation by CD28 of IL-2R and c-*myc* expression is uncertain. IL-2 itself increases IL-2R and c-*myc* expression (46); however, we observed potentiation of PMA-induced IL-2R and c-*myc* expression by CD28 before the appearance of IL-2 gene induction.

The IL-2 gene is expressed only in T lymphocytes, indicating the central role of T cells in cellular regulation. Many cell types express the receptor for IL-2, and moreover, IL-2 has been suggested to regulate such diverse functions as B-cell immunoglobulin synthesis (3), monocyte differentiation (24), and hematopoiesis (2). Our results are consistent with previous studies suggesting that IL-2 and IL-2R gene expression are independently regulated in T cells (12, 28, 39).

T-cell proliferation induced by CD28 plus PMA was entirely resistant to cyclosporine, in contrast to the sensitive pattern that we found for CD3 and that others have reported previously for CD3 and the lectins PHA and concanavalin A (14, 18, 30, 31, 64). An intermediate result was found with PMA or CD28 plus CD3-T-cell receptor-induced prolifera-

tion; neither signal was able to confer complete resistance to cyclosporine, although the addition of either CD28 or PMA to CD3-stimulated cultures resulted in less sensitivity to cyclosporine than after stimulation by immobilized CD3 alone. Moreover, we found that the IL-2 gene was fully expressed after CD28 plus PMA stimulation in the presence of cyclosporine, indicating that the mechanism of cyclosporine is not to prevent IL-2 expression at the level of mRNA transcription, as has been suggested (18, 30), but rather at an earlier step that is bypassed by CD28 stimulation.

We confirmed the observation of Geppart et al. (16) that stimulation of purified T cells with immobilized CD3 antibody causes full proliferation. In the present study we found that surprisingly small amounts of IL-2 mRNA were produced in cultures of purified T cells stimulated with CD3 and that CD28 caused a ~30-fold augmentation of IL-2 mRNA expression in cells stimulated with CD3. Others have shown that CD3 stimulation can be associated with lower than expected amounts of IL-2 secretion (28). Both Geppart et al. (16) and we (unpublished data) have found that CD28 stimulation does not augment proliferation (thymidine incorporation) of cells stimulated optimally with immobilized CD3 antibodies. It is important to note, however, that the present results (Fig. 7) indicate that the CD28 molecule still provides a signal in cells stimulated by immobilized CD3. Thus, the barely detectable levels of IL-2 mRNA after CD3 stimulation may reflect the amount of IL-2 that is necessary and sufficient for proliferation in an autocrine fashion, and therefore our results suggest that the role for CD28 may be to augment IL-2 production and thereby to recruit cells via a paracrine as well as an autocrine mechanism. CD28 would then have an effect to amplify a response that was initiated in an antigen-specific manner in vivo by upregulation of IL-2 production. Alternatively, the minimal amounts of IL-2 mRNA expressed after stimulation only by immobilized CD3 antibodies could indicate that CD3-T-cell receptor-induced proliferation is primarily dependent on growth factors other than IL-2.

One can speculate that the CD28 pathway may be important in certain states of immune dysregulation known to be resistant to treatment with cyclosporine. For example, cyclosporine has been very effective as an immunosuppressant in solid-organ transplantation, as it is able to prevent graft rejection in spite of major histocompatibility differences (52). In contrast, cyclosporine has been relatively ineffective as treatment for graft-versus-host disease following allogeneic bone marrow transplantation (54), a disease thought to be mediated by cytotoxic T lymphocytes that express the CD28 antigen (55). Previous studies have demonstrated that T-cell proliferation is inhibited in vitro by elevation of the cytoplasmic cAMP concentration and that the level of this inhibition is at an early step in activation that is distinct from that of cyclosporine (7, 43). We found that CD3-T-cell receptor-stimulated proliferation was inhibited by prostaglandin E2 and that the inhibition could be overcome by CD28 stimulation (36). In addition we have found that CD28 stimulation confers resistance to the antiproliferative effects of glucocorticoids (unpublished data). Thus, the CD28 pathway is remarkable in that it overcomes the effects of several distinct immunosuppressants, suggesting that the CD28 pathway may have an important role in vivo in T-cell proliferation that is resistant to immunosuppressants.

The biochemical events triggered by cellular activation through the CD3-T-cell receptor pathway and the CD2 sheep rosette receptor pathway appear to be identical (27, 44) and most probably differ functionally in that the CD3 pathway can activate T cells in an antigen-specific manner while the CD2 pathway, presumably through endogenous ligands such as LFA-3, can activate both T cells and large granular lymphocytes to proliferate in an antigen-nonspecific manner (27, 40, 48). Together, our results suggest that the CD28 pathway activates a major subset of T cells to proliferate via an IL-2-dependent mechanism that is distinct biochemically from CD2 and CD3.

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