CD28 Ligation in T-Cell Activation: Evidence for Two Signal Transduction Pathways

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The CD28 homodimer is thought to function as a signal transducing receptor during activation of T cells. Evidence is presented that the degree of aggregation of CD28 on the cell surface regulates two distinct CD28-associated signals. Binding of bivalent CD28 monoclonal antibody (MoAb) 9.3 upregulates lymphokine production by messenger RNA (mRNA) stabilization, without direct initiation of lymphokine mRNA transcription. This signal was not dependent on inositol phospholipid production or activation of a protein tyrosine kinase (PTK). In contrast, further crosslinking of CD28 on the cell surface rapidly induced formation of large amounts of inositol trisphosphate (InsP3) and increased cytoplasmic calcium concentration ([Ca2+]i), but did not stimulate PTK. CD28 crosslinking directly activated a subset of resting T cells, since CD25 (interleukin [IL]-2 receptor) mRNA was rapidly induced in purified T cells, and proliferation, even without addition of exogenous IL-2, was sometimes observed. CD25 expression was detected on the cell surface of approximately 20% of CD4+ T cells. The degree of CD28 aggregation required for activation was investigated by preparing soluble 9.3 x 9.3 conjugates ranging in size from approximately 300 Kd to greater than 1,000 Kd, and comparing their function in T-cell proliferation assays with phorbol-12-myristate-13-acetate (PMA), anti-CD3, or IL-2. There was a correlation between conjugate size and proliferation with IL-2, whereas costimulation with PMA or CD3 was optimized at a lower degree of CD28 aggregation. The inositol phospholipid (InsP) generation and increase in [Ca2+]i after CD28 receptor aggregation appeared to proceed through a pathway different from the CD3/T-cell receptor (TCR) pathway since it was enhanced by pretreatment with PMA, while the InsP and [Ca2+]i signal from crosslinking CD3 was suppressed by PMA. Furthermore, the proliferation response to CD28 aggregation was resistant to inhibition by CD3 modulation. Thus, CD28 aggregation appears to trigger a phospholipase C activation pathway that differs from the CD3/TCR-linked pathway.

MATERIALS AND METHODS

MoAbs and reagents. CD3 antibodies G19-4 (BALB/c, immunoglobulin [Ig]G1) and 38.1 (BALB/c, IgM), and CD4 MoAb G17-2 (BALB/c, IgG1), CD8 MoAb G10-1 (BALB/c, IgG2a), CD5 MoAb 10.2 (BALB/c, IgG2b), and CD28 MoAb 9.3 ([BALB/c x C57BL/6]F1, IgG2b) were previously described.6,17-19 These antibodies were purified from ascites fluid by protein A chromatography (6.1, 9.3, 10.2, and G10-1) or by salt precipitation and ion exchange chromatography (G19-4, G17-2), followed by dialysis and filtration through 0.45 μm filters before use. Rat anti-mouse x chain MoAb 187.12 was purified by affinity chromatography using mouse Ig coupled to Sepharose (Pharmacia, Piscataway, NJ). MoAbs were conjugated with biotin using biotin-succinimide (Sigma Chemical Co, St Louis, MO) as described.21

9.3 x 9.3 Conjugate information. Conjugates were prepared using maleimido-butyroluccin-mide (GMBS; Calbiochem, La

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Submitted August 3, 1989; accepted December 8, 1989.

Supported by Oncogen, National Institutes of Health Grant AG 01751, and NMRDC No. MM33C30.005-1000.

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Blood, Vol 75, No 7 (April 1), 1990: pp 1531-1539
1531
Jolla, CA) and immunoaffinity HCl (2-IT; Pierce Chemical Co, Rockford, IL) with a procedure similar to MAB-PE coupling. Briefly, one aliquot of 9.3 was treated with 2-IT at 250 µg/mg MoAb and a second aliquot of 9.3 was treated with GMBS at 7 µg/mg MoAb. Derivatized MoAbs were desalted and mixed together to form a stable thioether bond. MoAb conjugates were separated by size-exclusion chromatography using a Superose-6 (Pharmacia, Uppsala, Sweden) column (5 × 140 cm). Fractions corresponding to molecular weights (mol wts) of 150,000 to greater than 660,000 were collected.

**Measurement of [Ca²⁺]i.** [Ca²⁺]i responses were measured using indo-1 (Molecular Probes, Eugene, OR) and a model 50 H1/2150 flow cytometer (Ortho, Westwood, MA) as we have previously described. The histograms were analyzed by programs that calculated the mean indo-1 violet/blue fluorescence ratio versus time. In addition, the percentage of responding cells versus time was analyzed by programs that first determined the value of the indo-1 ratio that was 2 SDs above the ratio for control cells, and then plotted the percentage of cells above this threshold value versus time. There are 100 data points on the x (time) axis on all flow cytometric data. Human lymphocytes were purified from peripheral blood by centrifugation on Ficoll/Hypaque (Pharmacia), followed by adherence to nylon columns to remove the majority of monocytes. In some experiments, CD4 cells were analyzed by staining with phycoerythrin (PE)-conjugated 2H7 anti-CD20, FC-2 anti-CD16, and G10-1 anti-CD8, and gating on the unstained cells, which were greater than 95% CD4+ (reciprocal CD4). In other experiments, CD8 cells were analyzed by staining with PE-conjugated 2H7 anti-CD20, FC-2 anti-CD16, and G17-2 anti-CD4 and gating on unstained cells, which were greater than 85% CD8+ (reciprocal CD8).

**Cells.** Resting CD28+ T cells were purified as described elsewhere, and used for studies of phosphoinositide metabolism and Northern blot analysis. Briefly, peripheral blood lymphocytes were obtained by leukopheresis of laboratory personnel and Ficoll-Hypaque density gradient centrifugation. The CD28+ subset of T cells was then isolated by negative selection with magnetic bead particles (Advanced Magnetics Institute, Cambridge, MA), antibody, and then incubated with goat anti-mouse Ig-coated magnetic beads (Advanced Magnetics Institute, Cambridge, MA), to remove the majority of monocytes. In some experiments, CD20+ cells with saturating amounts of MoAb. This strategy takes advantage of the reciprocal and nonoverlapping distribution of the CD28 and CD11b surface antigens on resting peripheral blood lymphocytes. The cells were washed three times to remove unbound antibody, and then incubated with goat anti-mouse Ig-coated magnetic particles (Advanced Magnetics Institute, Cambridge, MA), and the bead-coated cells removed by magnetic separation. Typically, 500 × 10⁶ CD28+ T cells were recovered that were greater than 98% CD28+, as assessed by flow cytometry, and contained less than 0.1% monocytes as determined by staining for nonspecific esterase. The purified CD28+ T cells were unable to respond to phorbol-12-myristate-13-acetate (PMA) indicating a low level of monocyte contamination.

**Measurements of [³H]inositol phosphates (IPs).** [³H]IPs were measured in a slight modification of previous methodology. Briefly, purified T cells were resuspended at 2 × 10⁶ cells/mL in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (“medium”), 4 µg/mL PHA, and 40 µCi myo-[³H]inositol (Amersham Corp, Arlington Heights, IL), and incubated at 37°C in 5% CO₂ in air. After 72 hours, cells had progressed into early G1 in the absence of DNA synthesis, and the increased metabolic activity allowed better [³H]inositol labeling (data not shown). The cells were washed three times and resuspended at 5 × 10⁶ cells/mL in “medium” supplemented with 10 mmol/L LiCl. After a 20-minute incubation, cells were stimulated for the indicated time interval and then sedimented for 10 seconds in an Eppendorf 5414 centrifuge (Eppendorf, Geratebau, FRG). After aspiration of the medium, 1 mL of ice-cold 10% trichloroacetic acid (TCA) (wt/vol) was added to the cellular pellet. After removal of insoluble material with 900 × centrifugation for 5 minutes, the supernatant was extracted with 6 vol of diethylether and then neutralized.

The [³H]IPs were separated by anion-exchange chromatography using Dowex 1-X8 in formate form (100 to 200 mesh; Bio-Rad, Richmond, CA) and quantified by liquid scintillation spectroscopy in Bio-Safe 2 (Research Products, Mt Prospect, IL).

**Measurement of cell proliferation.** For studies of cell proliferation, peripheral blood mononuclear cells were depleted of adherent T cells (monocytes and B cells) by two passage cycles over nylon columns as described. These populations contained approximately 1% monocytes as determined by staining for nonspecific esterase. Cells were cultured in quadruplicate samples in flat-bottom, 96-well microtiter plates at 5 × 10⁶ cells/well in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (HyClone, Logan, UT). Cell proliferation was measured in a liquid scintillation counter after pulsing the cells for the last 6 hours of 3- or 4-day cultures with 1 µCi/well of [³H]thymidine (New England Nuclear Corp, Boston, MA).

**Northern blot (RNA blot) analysis.** CD28+ T cells purified by immunomagnetic removal of B cells, large granular lymphocytes (LGL), and monocytes, were cultured at 2 × 10⁷/mL in complete medium. The cells were harvested by centrifugation, and total cell RNA was extracted with guanidine isothiocyanate. The samples were equalized for ribosomal RNA (rRNA), and the equalization was confirmed by ethidium bromide staining of equal amounts of the RNA samples separated as a nondenaturing 1% agarose gel, as previously described. These equalized RNA samples were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose. Membranes were baked under vacuum for 2 hours and then prehybridized at 42°C in a solution containing 50% formamide, 5X standard sodium citrate (SSC) (1X SCC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 1X Denhardt solution, 25 mmol/L sodium phosphate (pH 6.5), and 250 µg of [³H]tymidine (Calbiochem) per milliliter. Hybridizations were performed for 16 to 20 hours under identical conditions, except for the addition of 10% dextran sulfate and 10⁶ cpm of the DNA probe per milliliter. DNA probes were labeled by nick translation to a specific activity of 3 × 10⁹ to 9 × 10⁶ cpm/µg. Membranes were washed briefly at room temperature after hybridization in 2X SSC containing 0.1% sodium dodecyl sulfate (SDS), and then twice for 30 minutes each at 56°C in 0.1X SCC containing 0.1X SSC-0.1% sodium dodecyl sulfate. The membranes were then air-dried and exposed to x-ray film (Kodak XAR-2, Rochester, NY) for 48 hours at −70°C with intensifying screens.

The [³P]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 after digestion of the plasmid in which they were propagated with appropriate restriction endonucleases. The IL-2 α-chain receptor probe is a 1.0-kilobase (kb) EcoRI-BamHI fragment derived from pII1R3, obtained from W. Greene as controls, the B-chain cDNA fragment was used as previously described.

**Activation of PTK.** PTK activation was measured by immunoblotting with a purified rabbit anti-phosphotyrosine antibody, prepared as previously described. To prepare samples, 5 × 10⁶ cells/sample were stimulated as indicated, rapidly pelleted by centrifugation, and lysed in 200 µL boiling SDS sample buffer containing 20 µmol/L orthovanadate. Samples were boiled for 5 minutes and stored at −70°C until analysis on 10% SDS polyacrylamide gels, and transferred to immobilon (Millipore Corp, Bedford, MA). Immunoblots were incubated for 3 hours with 0.25 µg/mL anti-phosphotyrosine, followed by washing and development with 1
μCi/mL high specific activity ¹²⁵I Protein A (ICN Pharmaceuticals, Irvine, CA). Prestained high molecular weight markers (Bethesda Research Laboratory) were run on each gel, and migration positions are indicated by arrows. The rabbit anti-phosphotyrosine antibody did not react with resting or activated T cells in the presence of 40 μmol/L of the hapten phenyl phosphate (data not shown).

RESULTS

The involvement of CD28 receptor aggregation in proliferation responses of resting peripheral blood T cells was investigated by using monovalent Fab fragments or bivalent F(ab')₂ fragments of MoAb 9.3 and additional crosslinking on the cell surface. Table 1 shows that 9.3 Fab was unable to synergize with PMA or IL-2, whereas 9.3 F(ab')₂ was costimulatory with PMA but not IL-2. When CD28 was further crosslinked on the cell surface by addition of 9.3 F(ab')₂, followed by MoAb 187.1 (rat anti-mouse k chain), the synergy with PMA was increased approximately 10-fold, and significant proliferation in the presence of IL-2 was observed.

To explore these observations in more detail, 9.3 was conjugated to itself (9.3 x 9.3) by a stable thioether linkage and then separated into distinct size fractions by chromatography on Superose 6 size-exclusion resin (Fig 1A). Three pools of high molecular weight (9.3 x 9.3) conjugates were collected, corresponding to approximate molecular weights of greater than 1,000,000 (pool 1), 600,000 (pool 2), and 300,000 (pool 3). These conjugates were then compared with 9.3 Fab fragments, 9.3 intact MoAb, and (9.3 x 9.3) pool 1 crosslinking with 187.1 in proliferation assays using purified T cells (Fig 1B). This experiment showed interesting differences in the requirement for CD28 aggregation in costimulation with immobilized anti-CD3, PMA, or IL-2. The degree of CD28 crosslinking correlated closely with proliferation in the presence of IL-2, and under conditions of extreme CD28 crosslinking ([9.3 x 9.3] pool 1 plus 187.1), proliferation of nylon wool enriched T cells could proceed even without exogenous IL-2. In contrast, CD28 synergy with immobilized anti-CD3 (20 μg/mL G19-4 coated on plastic) required bivalent 9.3 but was not further increased by (9.3 x 9.3) pools. Costimulation with PMA was enhanced by (9.3 x 9.3) pool 3 compared with bivalent 9.3, but was then at a plateau of maximum proliferation and did not further increase with additional CD28 crosslinking.

The above results show that the induction of responsiveness to IL-2 is related to the extent of CD28 crosslinking. To explore this further, T cells were examined by immunofluorescence for CD25 expression after CD28 crosslinking. CD25 expression was detected on day 2 or 3 after stimulation, and was restricted to approximately 15% to 20% of CD4⁺ T cells (data not shown). Induction of CD25 mRNA after CD28 crosslinking was also examined in experiments using purified T cells (Fig 2). These cell populations contained less than 0.1% monocytes (by nonspecific esterase staining), and were unable to proliferate in response to PHA, indicating that CD28 crosslinking could induce CD25 mRNA without requiring an additional monocyte-derived signal. Crosslinking CD28 either by 9.3 immobilized on plastic (Fig

Table 1. CD28 Crosslinking Is Required for Proliferation

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>None</th>
<th>1 ng/mL PMA</th>
<th>100 U/mL IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>9.3 Fab</td>
<td>0.2</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>9.3 Fab(λ)₂</td>
<td>0.3</td>
<td>23.7</td>
<td>0.8</td>
</tr>
<tr>
<td>9.3 Fab(λ)₂ + 187.1</td>
<td>1.3</td>
<td>238.0</td>
<td>35.8</td>
</tr>
<tr>
<td>187.1</td>
<td>0.6</td>
<td>0.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Peripheral blood T cells were isolated by two cycles of passage over nylon wool columns and stimulated as shown, using 9.3 Fab or Fab(λ)₂ at 1 μg/mL and 187.1 at 10 μg/mL. Cultures were in quadruplicate and SEMs were less than 11% at every point. Uptake of [³H]thymidine was measured during the last 6 hours of a 4-day experiment. Representative of three experiments.
IL-2 R

Fig 2. Effect of CD28 aggregation on IL-2 receptor α chain mRNA expression. CD28 + T cells were cultured in the presence of media alone; anti-CD28 MoAb 9.3 (immobilized to plastic) for 1, 4, or 23 hours; anti-CD5 MoAb 10.2 (immobilized to plastic) for 1, 4, or 23 hours; or PHA plus PMA control. The top panel shows the hybridization with IL-2R α-specific probe, whereas the bottom panel represents a duplicate blot hybridized with a β-actin probe. One of two similar experiments.

2) or by using 9.3 in solution followed by 187.1 anti-κ was effective (data not shown). CD25 mRNA was seen at 4 and 23 hours after CD28 crosslinking. In contrast, CD5 crosslinking induced transient expression of CD25 mRNA detectable at 1 hour but not at 4 hours (Fig 2).

CD28 crosslinking was previously reported to increase [Ca²⁺]i in T cells. Figure 3 shows that the effect of CD28 crosslinking on [Ca²⁺]i was predominantly among CD4⁺ T cells, and thus was similar to the response from anti-CD3. Although CD28 is expressed by greater than 50% of CD8⁺ cells, their response was barely detectable. The increase in [Ca²⁺]i among CD4⁺ cells was detectable in the absence of extracellular calcium (chelated with 10 mmol/L EGTA), indicating that mobilization of cytoplasmic calcium had occurred. CD28 receptor aggregation in Fig 3 depended on crosslinking of biotin-conjugated 9.3 MoAb on the cell surface by the addition of avidin. In numerous experiments with either resting or preactivated T cells, the bivalent 9.3 MoAb has not been seen to increase [Ca²⁺]i without additional crosslinking (data not shown). However, the high mol wt (9.3 x 9.3) conjugates were effective in calcium mobilization with slower kinetics. Figure 4 illustrates that (9.3 x 9.3) pool 1 had increased [Ca²⁺]i in a significant proportion of T cells at 30 minutes after stimulation, whereas cells stimulated with bivalent 9.3 were not significantly different from unstimulated cells. At 30 minutes after stimulation, CD28 receptor aggregation with either (9.3 x 9.3) pool 1 or with 9.3 plus 187.1 were similar in their effect on [Ca²⁺]i (Fig 4).

The above-mentioned data may explain conflicting results previously reported, since it is likely that some studies used anti-CD28 that contained aggregates capable of enhanced crosslinking.

The increase in [Ca²⁺]i that results from InsP3 formation has been found, in several cases, to be inhibited by treatment of cells with PMA. Thus, the coupling to InsP3 formation and calcium mobilization by the slg receptor and CD19 receptor of B cells10,11 and the CD3/Ti receptor and CD5 receptor of T cells12,13 are rapidly downregulated by the action of protein kinase C. The signal linked to aggregation of the CD28 receptor was found to be different since the increase in [Ca²⁺]i was augmented by pretreatment with PMA (Fig 5). In this experiment, the time of pretreatment...
with PMA (10 ng/mL) was varied and the effect of CD3-induced or CD28-induced increases in \([\text{Ca}^{2+}]_i\) was compared. By 1 hour after PMA addition, the CD3 signal was maximally suppressed whereas the CD28 signal was augmented by 50%. In other experiments, augmentation of the CD28 signal has been seen as early as 10 minutes after PMA treatment (data not shown). By 3 hours, the CD3 signal had recovered to control levels, but the CD28 signal was twofold above control.

Overnight PMA pretreatment resulted in a sixfold augmentation of \([\text{Ca}^{2+}]_i\) increases from CD28 receptor aggregation, but only a 50% increase in the CD3-mediated signal (Fig 5). The potentiation of the \([\text{Ca}^{2+}]_i\) increase coupled to CD28 receptor stimulation was derived from cytoplasmic sources of calcium, since the effect of PMA pretreatment was observed in the absence of extracellular calcium (10 mmol/L EGTA, Fig 6). Figure 6 also illustrates that PMA pretreatment augmented both the peak \([\text{Ca}^{2+}]_i\) increase and accelerated the kinetics of the response to CD28 crosslinking.

The involvement of inositol phospholipids in the increase in \([\text{Ca}^{2+}]_i\) after CD28 receptor aggregation was investigated using CD28+ T cells. The results (Fig 7) demonstrated that bivalent binding of 9.3, either in the presence or absence of PMA, did not increase InsP1, InsP2, or InsP3. This is consistent with the previous results demonstrating that calcium mobilization is not involved in the signal derived from soluble 9.3 MoAb.29 When biotin-conjugated 9.3 was further crosslinked on the cell surface by the addition of avidin, a substantial production of InsP1, InsP2, and InsP3 occurred. The response was sustained for greater than 15 minutes and was similar or possibly larger than would be expected from optimal stimulation of CD3. Pretreatment of the cells with PMA for 5 minutes accelerated the kinetics of the response to CD28 crosslinking.

Activation of PTK is an early event in signal transduction after antigen or anti-CD3 stimulation of T cells.34,35 To determine whether CD28 receptor can activate PTK, T-cell blasts were generated by stimulation for 72 hours and assayed for tyrosine-phosphorylated proteins by immunoblotting with rabbit anti-phosphotyrosine.29 Figure 8 shows that compared with resting T cells (lane 1) or IL-2 alone (lane 2), cells stimulated with immobilized anti-CD3 plus IL-2 (lane 3) were highly induced for PTK activity. Proteins (phosphorylated on tyrosine) of 122, 75, 62, 55, 51, 31, 29, and 27 Kd were most distinct. Cells stimulated with crosslinked CD2 + CD28 (lane 4) were similar except for a new 125-Kd protein not seen except in cells generated by PHA plus IL-2 (lane 7). When cells were stimulated by immobilized anti-CD3 plus CD28 MoAb in solution, there was an additional band at 88 Kd, and the 29-Kd protein was much stronger (lane 5). Again, only the PHA + IL-2 blasts (lane 7) contained similar amounts of these bands. However, cells induced by CD28 crosslinking + IL-2 (lane 6) were similar to resting cells in tyrosine-phosphorylated proteins.

This experiment suggested that CD28 crosslinking did not directly induce PTK, but that immobilized CD3 + CD28 MoAb in solution did upregulate PTK activity. This was most likely an indirect effect of CD28 stimulation, since IL-2 has been reported to signal through activation of PTK.36,37 and CD28 stimulation upregulates IL-2 production.9 To test
Control cells, but can upregulate PTK indirectly through induction of CD2. Thus, proliferation that occurs after stimulation with anti-CD2 MoAbs is inhibited by anti-CD3, suggesting that CD2 may be regulated by CD3. To examine whether the proliferation in response to CD28 aggregation plus IL-2 is also inhibited by CD3 internalization, the 38.1 (CD3, IgM) MoAb was used. Table 2 shows that PHA-induced proliferation in the presence of IL-2 was inhibited by 38.1 when purified T cells were cultured. CD28 aggregation either with 9.3 plus 187.1 or (9.3 \times 9.3) conjugate led to proliferation in the presence of IL-2 that was resistant to inhibition by 38.1. Similar results were obtained when the T-cell blasts or peripheral blood mononuclear cells were used (data not shown). Furthermore, CD2-induced proliferation was inhibited by 38.1 in experiments where CD28-induced proliferation was not. Thus, the CD28 pathway may differ from the PHA or CD2 pathway in its regulation by CD3.

**DISCUSSION**

The CD28 homodimer is a member of the Ig gene super family and is expressed by T cells that have TcR \( \alpha, \beta \) receptors, including most CD4+ cells and CD8+ cells that are CD11b−. Considerable evidence has been presented to show that CD28 MoAb 9.3 can deliver an important regulatory signal in T-cell activation. In costimulation assays with anti-CD3, PMA, or PHA, soluble 9.3 upregulates T-cell proliferation through a mechanism that acts primarily on inducible lymphokine mRNA to result in stabilization of these otherwise labile mRNAs. The nature of the biochemical signal associated with bivalent binding of 9.3 is controversial, since increases in \([Ca^{2+}]\) have been reported in some but not other studies. Here we present evidence that soluble 9.3 does not initiate inositol phospholipid production or activate PTK. These results are consistent with our previous demonstration that soluble 9.3 upregulates IL-2 mRNA levels induced by optimal concentrations of PMA and ionomycin, and therefore activates a pathway other than the inositol phospholipid pathway. The rapid four- to sixfold increase in cGMP may represent the biochemical basis for transmembrane signaling by the CD28 receptor after bivalent binding of 9.3.

Our data show that a second transmembrane signal associated with CD28 is dependent on a higher degree of receptor aggregation. Crosslinking of CD28 MoAb 9.3 does induce rapid production of large amounts of inositol phospholipids and mobilizes cytoplasmic calcium through a pathway that is enhanced rather than suppressed by PMA. This CD28 signal depends on crosslinking biotin-conjugated 9.3 MoAb on the cell surface with avidin or a second-step anti- \( \alpha \) MoAb such as 187.1. Alternatively, high mol wt conjugates (9.3 \times 9.3) were also able to induce the signal associated with CD28 aggregation, and their effectiveness was dependent on their size. The cellular response after CD28 crosslinking included direct induction of CD25 mRNA by purified T cells, and proliferation in the presence of IL-2 without the requirement for a signal from other receptors such as CD2 or CD3. Previous evidence that either monocytes or monocyte-derived IL-6 was required to induce responsiveness to IL-2 after CD28 crosslinking may have resulted from the conditions of crosslinking used (immobilized 9.3). Our data demonstrate a direct correlation between the degree of CD28 crosslinking and the amount of responsiveness to IL-2 by purified T cells (Fig 1). This hypothesis of the transmembrane signaling pathways regulated by the CD28 receptor is shown in Fig 10.
In the absence of other signals, CD28 aggregation alone did not directly induce IL-2 mRNA (data not shown). However, we have found that under the most extreme conditions of CD28 aggregation using a (9.3 × 9.3) conjugate composed of 6 to 8 MoAbs followed by 187.1 (anti-CD3 MoAb), proliferation of purified T cells occurred without requiring addition of exogenous IL-2 (Fig 1). Thus, further experiments are required to determine whether direct induction of lymphokine genes may occur by a high degree of CD28 aggregation.

The inositol phospholipid production and increase in \([\text{Ca}^{2+}]_i\) after CD28 crosslinking may occur through a biochemical pathway distinct from the CD3- or CD2-associated pathways. The evidence for this is indirect but includes: (1) treatment of T cells with PMA rapidly upregulates the calcium signal associated with CD28 but inhibits the signals associated with CD3 (Fig 5), CD2 (data not shown), or CD5. Thus, the CD28 pathway may have a unique interaction with protein kinase C or a substrate of protein kinase C. (2) The proliferation of purified T cells in the presence of exogenous IL-2 after CD28 aggregation was not susceptible to inhibition by an IgM anti-CD3 MoAb 38.1 (Table 2). This is unlike proliferation induced by anti-CD2

**Table 2. CD28 Activation Pathway Is Not Inhibited by CD3 MoAb 38.1**

<table>
<thead>
<tr>
<th>Proliferation in the Presence of 100 U/mL IL-2 + 187.1</th>
<th>(^{3}H)-thymidine Incorporation, cpm (\times 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 MoAb % Change</td>
<td>CD3 MoAb % Change</td>
</tr>
<tr>
<td>Proliferation Signal</td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>5.7</td>
</tr>
<tr>
<td>PHA (1 pg/mL)</td>
<td>427.6</td>
</tr>
<tr>
<td>CD28 (9.3 × 9.3)</td>
<td>20.6</td>
</tr>
<tr>
<td>CD28 (9.3 + 187.1)</td>
<td>49.1</td>
</tr>
<tr>
<td>PMA (1 ng/mL)</td>
<td>32.2</td>
</tr>
</tbody>
</table>

T cells were prepared by two cycles of passage over nylon wool columns and stimulated as indicated using all MoAb or MoAb conjugates at 1 µg/mL and 187.1 anti-CD MoAb at a fourfold excess. Proliferation was measured in quadruplicate cultures by uptake of \(^{3}H\)-thymidine during the last 6 hours of a 3-day experiment. Means are shown, and SEs were less than 15% of the mean. Representative of six separate experiments.
molecularly interact with other surface receptors in a fashion similar to the CD4 or CD8 interactions with CD3/Ti. Furthermore, the CD28 pathway leading to activation of lymphokine genes is unique in its resistance to immunosuppressants such as cyclosporine, and prostaglandin E. (4) CD28 crosslinking initiates production of large amounts of inositol phospholipids but does not activate protein tyrosine kinase, whereas both CD2 and CD3 pathways activate both inositol phospholipid and PTK responses. T-cell blasts induced by costimulation with anti-CD3 plus anti-CD28 contain higher levels of tyrosine-phosphorylated proteins than blasts induced with anti-CD3 alone; however, this is most likely an indirect effect of anti-CD28 caused by induction of higher levels of IL-2, which directly activate tyrosine kinase(s) in T cells.

Multiple evidence now points to the central role of CD28 aggregation in regulating the signals delivered by the 9.3 MoAb. For example, the inhibition of antigen-specific stimulation by soluble 9.3 was reversed by additional crosslinking on the cell surface, suggesting that CD28 aggregation may be more important in antigen-specific responses than in responses to polyclonal mitogens such as PHA, PMA, or anti-CD3. It is noteworthy that receptors on nonlymphoid cells, such as the epidermal growth factor (EGF) receptor and the insulin receptor, may also be regulated by aggregation. Receptor aggregation induced by ligand binding has recently been shown for insulin receptors. Other studies with antibodies to EGF receptors or insulin receptors showed that MoAb crosslinking could activate the receptors. Thus, the discovery of the ligand for the CD28 receptor is critical for further insights regarding the physiologic importance of CD28 aggregation.

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CD28 ligation in T-cell activation: evidence for two signal transduction pathways

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