Immunology 1998 **93** 358–365

Simultaneous cross-linking of CD6 and CD28 induces cell proliferation in resting T cells

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SUMMARY

In the present study, we showed that simultaneous ligation of the monoclonal antibodies (mAb) against CD6 and CD28 induces T-cell proliferation in purified resting T lymphocytes in the absence of T-cell receptor (TCR) occupancy. No cell proliferation was observed when the mAb were cross-linked alone or used simultaneously in the soluble form. T-cell proliferation mediated through CD6/CD28 is accompanied by the up-regulation of interleukin-2 (IL-2) mRNA and expression of IL-2 receptors on the cell surface. In the presence of IL-2-neutralizing mAb the proliferative response of the T cells induced through CD6/CD28 was inhibited dose dependently. Cross-linking mAb to CD6 and CD28 alone or together did not down-regulate the CD3/TCR complex. T-cell proliferation mediated through CD6/CD28 was only partially blocked by the immunosuppressive drug, cyclosporin A (CsA), whereas anti-CD28-induced T-cell proliferation in the presence of the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was unaffected. In sharp contrast T-cell proliferation mediated by anti-CD6 in the presence of TPA was efficiently blocked by CsA. In addition, two protein kinase C (PKC) inhibitors, GF 109203X and H-7 dose-dependently inhibited T-cell proliferation mediated through CD6/CD28, suggesting that PKC activation may be involved. Furthermore, there was a marked differential dose-dependent inhibitory effect of the PKC inhibitors on T-cell proliferation mediated by the co-ligation of anti-CD6 or anti-CD28 in the presence of anti-CD3, with the former being more sensitive to PKC inhibition. Taken collectively, our results suggest that T-cell activation can occur through an antigen-independent pathway by cross-linking the accessory molecules, CD6 and CD28, and that these two cell surface antigens may have distinct signalling pathways.

INTRODUCTION

It is well established that optimal T-cell activation leading to cell proliferation requires at least two stimulatory signals. One of these signals is delivered by the T-cell receptor (TCR), which recognizes antigen in the form of peptide fragments bound to either major histocompatibility complex (MHC) class I or class II molecules on antigen-presenting cells (APC). The second signal, which is not antigen-specific, has been termed the co-stimulatory or accessory signal, because, while essential, it does not by itself induce any proliferative response in resting T cells.[1,2] In recent years several potential co-stimulatory signal receptors and their ligands have been identified, and these include the interactions between CD4 with class II MHC, CD8 with class I MHC, CD2 with lymphocyte function-associated antigen-3, CD5 with CD72 and CD28 with B7-1/B7-2.[2,3]

It is well established that the cell surface molecule, CD6 can act as accessory receptor and is capable of providing the co-stimulatory signals needed to synergize with the signals mediated through the TCR to enhance T-cell proliferation.[4–6] The CD6 cell surface molecule is a membrane glycoprotein expressed mainly on mature T cells, medullary thymocytes and a subpopulation of B cells.[7–9] Its ligand, known as ALCAM, has recently been cloned and characterized,[10–12] and was found to be highly expressed in thymic epithelial (TE) cells, activated T cells, B cells and monocytes.[10–12] It is now apparent that the binding of thymocytes to TE cells is in part mediated by the CD6/ALCAM interactions, suggesting that CD6 may play a major role in regulating T-cell development and maturation.[12] Although cross-linking monoclonal antibodies (mAb) against the CD6 molecule have been shown to up-regulate interleukin-2 (IL-2) mRNA and to augment the T-cell proliferation induced by anti-CD3 mAb or 12-*O*-tetradecanoylphorbol-13-acetate (TPA),[4–6,13,14] the signalling pathway of CD6 is still unclear. Recently, we provided evidence

Received 4 September 1997; revised 30 November 1997; accepted 30 November 1997.

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PKC, protein kinase C; PTK, protein tyrosine kinase; RAM, rabbit anti-mouse; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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that a protein-tyrosine kinase (PTK) is rapidly activated after cross-linking the CD6 receptor, and the abrogation of this PTK activity completely blocked T-cell proliferation,[14] suggesting that the activation of tyrosine kinase formed part of the signal transduction pathway mediated through CD6. Similarly, PTK activation also forms part of the signalling mechanisms of another accessory molecule, CD28. Furthermore, it is now known that activation via CD28 also involves phosphoinositide 3-kinase (PI3-kinase) activation, and is distinct from that mediated through the TCR/CD3 complex.[3,15,16] The signals delivered by the interactions between CD28 and its ligand, B7, appear to involve the stabilization of mRNA for various lymphokines, followed by an increase in transcription.[17] Although the signalling pathway of CD6 is less well characterized compared to that of CD28, there are some similarities between the effects of these two cell surface antigens. For instance, ligation of either one of the two receptors synergized with signals via the TCR complex to enhance T-cell proliferation.[4,6,18] They are also capable of triggering T-cell proliferation in the presence of TPA resulting in the up-regulation of IL-2 receptors (IL-2R) and IL-2 mRNA.[14,19,20] Both receptors when cross-linked also induced a moderate increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in T lymphocytes,[20,21] and finally both receptors involved PTK in their signalling mechanism.[14,20]

To examine further the signalling pathways involved during the ligation of CD6 and to explore the biological importance of simultaneous presentation of CD6 and CD28 ligands, we examined the effect of cross-linking mAb to CD6 and CD28 by rabbit anti-mouse (RAM) immunoglobulins on cell proliferation in purified T cells. Our results indicate that simultaneous ligation of CD6 and CD28 in the absence of TCR/CD3 occupancy induced an IL-2-dependent T-cell proliferation which was partially sensitive to cyclosporin A.

MATERIALS AND METHODS

Monoclonal antibodies and other reagents

The anti-CD6 mAb, IOR-T1 (IgG2a, clone F5/43/27/F6) was produced as previously described.[6] Anti-CD28 mAb (IgG1, clone CD28.2) was from Immunotech (Luton, UK). The anti-CD3 mAb, OKT3 (IgG2a), was purified from hybridoma supernatant (American Type Culture Collection, Rockville, MD). Phycoerythrin (PE)-labelled CD25 mAb (IL-2 receptor) was from Beckton Dickinson (Mountain View, CA). Anti-Human IL-2 mAb was a gift for Dr M. Araña (CIGB, Havana, Cuba). RAM immunoglobulins and PE-labelled anti-CD3 mAb were obtained from Dakopatts (Copenhagen, Denmark). TPA was obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll–Hypaque was from Pharmacia (Uppsala, Sweden). RPMI-1640 was purchased from Gibco BRL (Gaithersburg, MD). Dynabeads coupled with anti-CD4 or anti-CD8 mAb, and Detachabead were obtained from Dynal A.S. (Oslo, Norway). Cyclosporin A (CsA) was a generous gift from Sandoz (Basel, Switzerland). H-7 and GF 109203X were from Calbiochem-Novabiochem Co. (Nottingham, UK).

T-cell separation

Naive $CD4^+$ and $CD8^+$ cells were purified as previously described.[14] In brief, human peripheral blood mononuclear cells (PBMC) from venous blood samples from healthy adult volunteers were obtained by Ficoll–Hypaque density gradient centrifugation. The PBMC were incubated with Dynabeads coated with anti-CD4 or anti-CD8 mAb for 60 min at 4°. The bead-coated cells were incubated with Detachabead for 45 min at room temperature and the detached cells were recovered after removing the beads with the magnetic particle concentrator. This isolation procedure routinely yielded a population of T cells that was 99% $CD3^+$ as assessed by flow cytometry.

Proliferation assay

Purified T cells (1×10^5 cells/well) in 200 µl of RPMI-1640 supplemented with 10% AB^+ human serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) were cultured in flat-bottom 96-well plates (Costar, Cambridge, MA) for 72 hr at 37° under an atmosphere of 5% CO_2 in air. Cells were activated with IOR-T1 (1 µg/ml), anti-CD28 (10 µg/ml), OKT3 (1 µg/ml), or TPA (4 ng/ml) alone or in combination as indicated. To cross-link the mAb, culture plates were preincubated overnight at 4° with RAM immunoglobulins (10 µg/ml) in 0·05 M bicarbonate buffer (pH 9·6). Unbound antibodies were removed by washing with phosphate-buffered saline (PBS). In some cultures CsA was used at 0·5 and 1 µg/ml. When indicated, the protein kinase C (PKC) inhibitors, GF 109203X and H-7 were used at 0·12–0·5 µM and 12·5–50 µM, respectively. The cells were pulsed for the last 18 hr with 1 µCi/well of [^{3}H]thymidine and then harvested onto glass fibre filters (Skartron Inc., Lier, Norway). The filters were dried and radioactivity was determined using liquid scintillation counting.

Immunofluorescence studies

T cells were cultured in RAM immunoglobulin-coated 96-well plates in the presence or absence of IOR-T1, anti-CD28 or TPA, alone or in combination. After 24 hr the cells were collected and stained with PE-labelled CD25 mAb (IL-2 receptor) for 30 min at 4°, before flow cytometry analysis using a fluorescence-activated cell sorter (FACScan) equipped with an argon laser (Beckton Dickinson). Forward- and side-scatter gatings were set to include lymphocytes and lymphoblasts. For the modulation CD3/TCR complex experiments, T cells were incubated with mAb to CD6, CD28 alone or both together followed by anti-mouse at 37° for 60 min. The expression of the CD3/TCR complex were determined using PE-conjugated anti-CD3 mAb staining followed by flow cytometry analysis. A total of 1×10^4 cells of the gated population were analysed.

RNA extraction and polymerase chain reaction amplification of mRNA

Total RNA were extracted from 10×10^6 cells as previously described.[22] The mRNA levels of IL-2 were analysed using the reversed transcription–polymerase chain reaction (RT-PCR) described elsewhere.[23] In brief, total RNA were denatured at 90° for 5 min and quickly chilled on ice. First-strand cDNA was generated using random hexanucleotides (Pharmacia-LKB, Uppsala, Sweden) and reverse transcriptase (BRL, Life Technologies, Inc., MD). PCR amplification of the resultant cDNA was carried out in a final volume of 20 µl containing 2 µl of 10× PCR buffer (Boeringher Mannheim, Germany), 1 µl dNTP (5 mM each), 2 µl of 5 µM IL-2 or the control gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) specific primers, 0·1 µl (5 U/µl) of Taq polymerase

(Boeringher Mannheim), 1 μl of the first-strand cDNA and water. PCR conditions were 1 min at 94° for denaturation, 1 min at 55° for annealing, 1 min at 72° for primer extension and amplification for 30 cycles. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide before being visualized using ultraviolet illumination. The oligonucleotide primers used were:
IL-2, 5′-TGTACAGGATGCAACTCCTG (sense), and 5′-CA-ATGGTTGCTGTCTCATCAG (antisense); and G3PDH, 5′-TGAAGGTCGGAGTCAACGGATTTGGT (sense), and 5′-CATGTGGGCCATGAGGTCCACCAC (antisense) which were generously provided by Dr Dude Gigliotti (Stockholm, Sweden). The resultant PCR products were 400 base pairs (bp) for IL-2 and 983 bp for G3PDH.

RESULTS

Cross-linking of CD6 and CD28 molecules induces T-cell proliferation

In order to mimic the physiological situation of binding to cell-bound ligands, purified resting T cells were cultured for 3 days on RAM immunoglobulin-coated plates with mAb against CD6 (IOR-T1) and CD28 molecules, either alone or together. As illustrated in Fig. 1, neither the cross-linking of the anti-CD6 mAb alone nor the anti-CD28 mAb by itself induced any T-cell proliferation which is in good agreement with previous reports.[6,24] However, when both anti-CD6 and anti-CD28 were cross-linked together with immobilized RAM

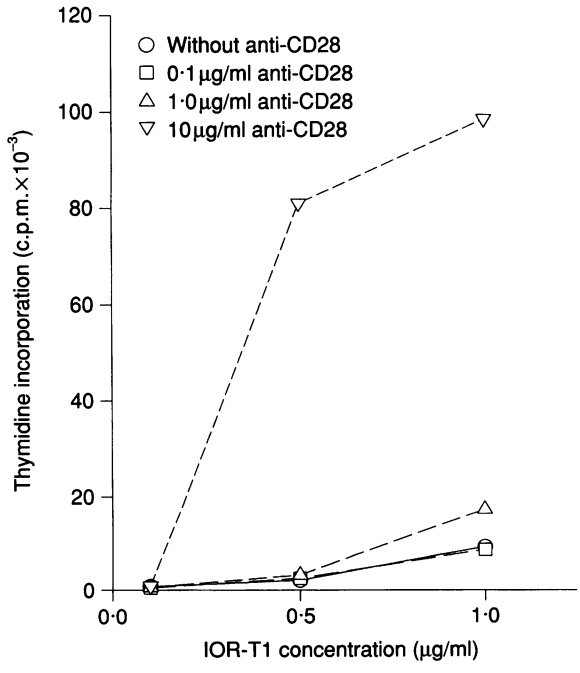

Figure 1. Dose response of T-cell proliferation induced by cross-linking mAb to CD6 and CD28 receptors. Purified T lymphocytes (1×10^5/well) were incubated with or without the anti-CD6 mAb, IOR-T1, and anti-CD28 mAb at different concentrations where indicated on RAM immunoglobulin-coated 96-well plates. Proliferation in triplicate cultures was determined after 72 hr by [^{3}H]thymidine incorporation, and results are expressed as the mean counts per minute (c.p.m.) of triplicate cultures.

immunoglobulin, a marked induction in cell proliferation was observed with the purified resting T lymphocytes (Fig. 1). Soluble mAb to CD6 and CD28 together have no effect on the resting T cells (results not shown). Dose–response studies showed that the CD6/CD28-induced T-cell proliferation was obtained only when more than 1 μg/ml of anti-CD28 was used, whereas 0·5 μg/ml of IOR-T1 mAb was adequate to induce the proliferation (Fig. 1). The optimal concentrations of 10 μg/ml for anti-CD28 and 1 μg/ml for IOR-T1 mAb was used in the subsequent experiments.

Co-stimulation by anti-CD6 and anti-CD28 mAb is mediated via an IL-2-dependent pathway

We have previously demonstrated that IL-2 mRNA was up-regulated in T cells when activated by the cross-linking of anti-CD6 in the presence of the phorbol ester, TPA, suggesting that CD6/TPA-induced T-cell proliferation may be IL-2-dependent.[14] We therefore investigated whether IL-2 was involved in the induction of the T-cell proliferation observed through the simultaneous cross-linking of anti-CD6 and anti-CD28 mAb. As illustrated in Fig. 2, after 24 hr of activation by cross-linking of IOR-T1 and anti-CD28 mAb, the p55 chain of the IL-2 receptor (CD25) was detected on almost 95·5% of the activated T cells. This high level of CD25 expression was equivalent to that observed in cells stimulated by cross-linking of IOR-T1 or anti-CD28 mAb alone in the presence of TPA. However, resting T cells stimulated by cross-linking of anti-CD28 alone with RAM immunoglobulin did not express IL-2 receptor above control levels (Fig. 2) whereas about 60% of the T cells expressed CD25 when stimulated with the cross-linking of IOR-T1 with RAM immunoglobulin. The latter, although having a greater number of cells expressing CD25, did not proliferate as indicated by the lack of [^{3}H]thymidine uptake under identical conditions (Figs 1 and 2). We next examined whether CD6/CD28 co-stimulation could induce the activation of the IL-2 gene and up-regulate the level of IL-2 mRNA. As shown in Fig. 3, purified T cells stimulated by the cross-linking of IOR-T1 and anti-CD28 mAb with RAM immunoglobulin had an increase in the expression of IL-2 mRNA (Fig. 3, lane 5). As expected, T cells stimulated with IOR-T1/TPA, CD28/TPA and OKT3/TPA had an increase in the expression level of IL-2 mRNA (Fig. 3, lanes 6, 7 and 8, respectively). Interestingly, T cells stimulated with IOR-T1 alone, which express IL-2 receptors but do not proliferate, had low or undetectable IL-2 mRNA (Fig. 3, lane 4). Little or no IL-2 mRNA was detected in T cells stimulated with TPA or anti-CD28 alone (Fig. 3, lanes 2 and 3, respectively). We next examined whether CD6/CD28-induced T-cell proliferation was dependent on IL-2. Using neutralizing antibodies to human IL-2, the T-cell proliferation induced by cross-linking CD6/CD28 was inhibited in a dose-dependent manner as illustrated in Fig. 4. Cell proliferation was unaffected in the presence of control mouse IgG mAb. Thus, CD6/CD28-stimulated T-cell proliferation is via an IL-2-dependent autocrine pathway.

Cross-linking of CD6 and CD28 does not down-regulate the CD3/TCR complex

It has been suggested that some cell surface receptors, such as CD4, may physically associate with the CD3/TCR complex

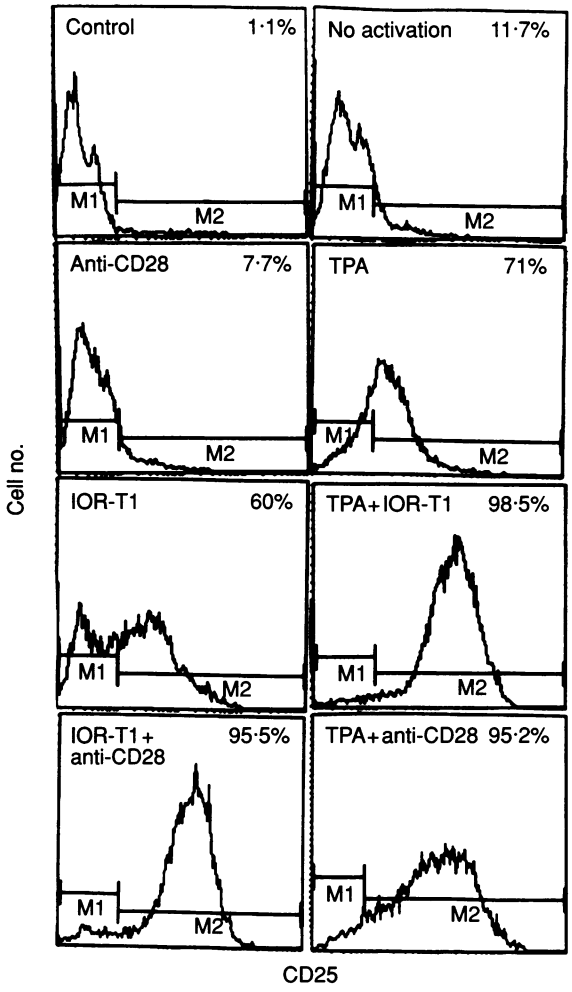

Figure 2. IL-2 receptor expression induced by simultaneous ligation of CD6 and CD28. Purified T cells were incubated in RAM immunoglobulin-coated 96-well plates in the presence or the absence of IOR-T1 (1 μg/ml), anti-CD28 mAb (10 μg/ml) or TPA (4 ng/ml) alone or in combination as indicated. After 24 hr, the cells were stained with PE-conjugated anti-CD25 (IL-2 receptor) and analysed by flow cytometry. An unrelated mAb conjugated with PE was used as control. The percentage of positive cells corresponding to the region M_2 is shown in the upper right corner.

as co-receptors, and are usually many fold more potent in activating T cells when cross-linked with the TCR.[25] To examine whether the CD6/CD28-induced T-cell proliferation after cross-linking with RAM immunoglobulin involves aggregation of the TCR, T cells were incubated with mAb to CD6 and CD28 followed by simultaneous cross-linking of the mAb and the co-capping of CD3/TCR was examined. As shown in Fig. 5 the simultaneous cross-linking of both CD6 and CD28 or on their own have little effect on the TCR expression in the cells, whereas OKT3 (anti-CD3 mAb) incubation resulted in rapid down-regulation of the TCR in the T cells. These findings suggest that the CD3/TCR complex is not physically associated with either CD6 or CD28, and that cross-linking with RAM immunoglobulin did not cause non-specific aggregation of the TCR/CD3 complex which may have contributed to the activation of T cells and proliferation.

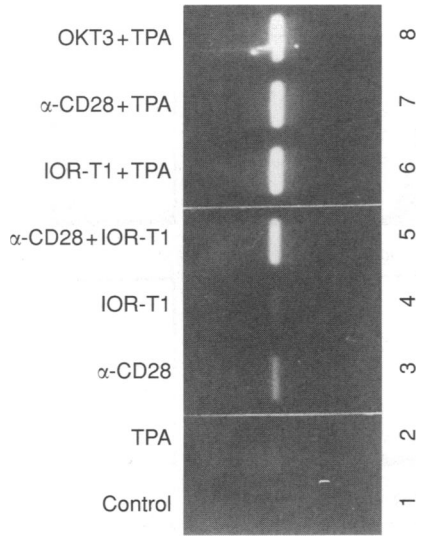

Figure 3. Induction of IL-2 mRNA by co-ligation of CD6 and CD28 receptors. Purified T cells (10×10^6 cells/well) were incubated in RAM immunoglobulin-coated 24-well plates with the following treatments: lane 1, control; lane 2, TPA (4 ng/ml); lane 3, anti-CD28 mAb (10 μg/ml); lane 4, IOR-T1 (1 μg/ml); lane 5, anti-CD28 plus IOR-T1; lane 6, IOR-T1 plus TPA; lane 7, anti-CD28 plus TPA; lane 8, OKT3 (1 μg/ml) plus TPA. After 18 hr of incubation, the total RNA was isolated, reverse transcribed and the cDNA was amplified by PCR, using IL-2-specific primers, as described in the Materials and Methods.

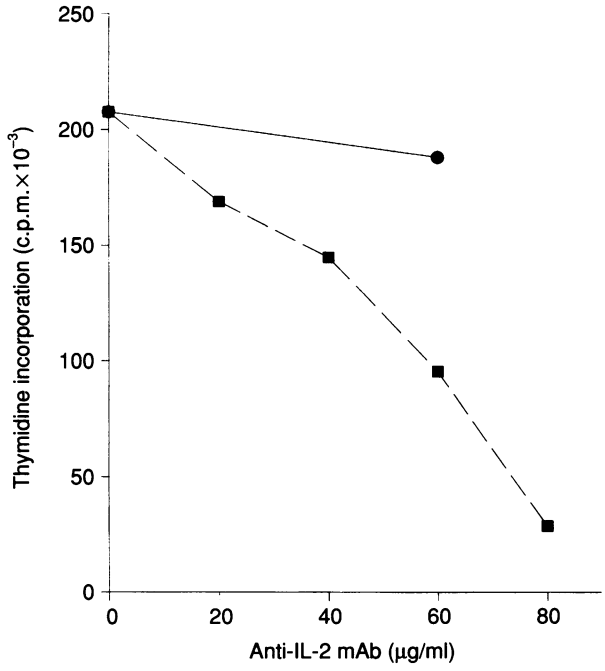

Figure 4. Inhibition of T-cell proliferation induced by cross-linking of CD6 and CD28 by neutralizing anti-human IL-2 mAb. Purified T cells (1×10^5/well) were incubated for 72 hr with IOR-T1 (1 μg/ml) and anti-CD28 mAb (10 μg/ml) together on RAM immunoglobulin-coated 96-well plates, with or without various concentrations of neutralizing anti-human IL-2 mAb (dashed line) or a control mouse IgG mAb (solid line). Proliferative responses were determined by [^{3}H]thymidine incorporation after 72 hr and results are expressed as the mean counts per minute (c.p.m.) from triplicate cultures from a representative experiment out of three.

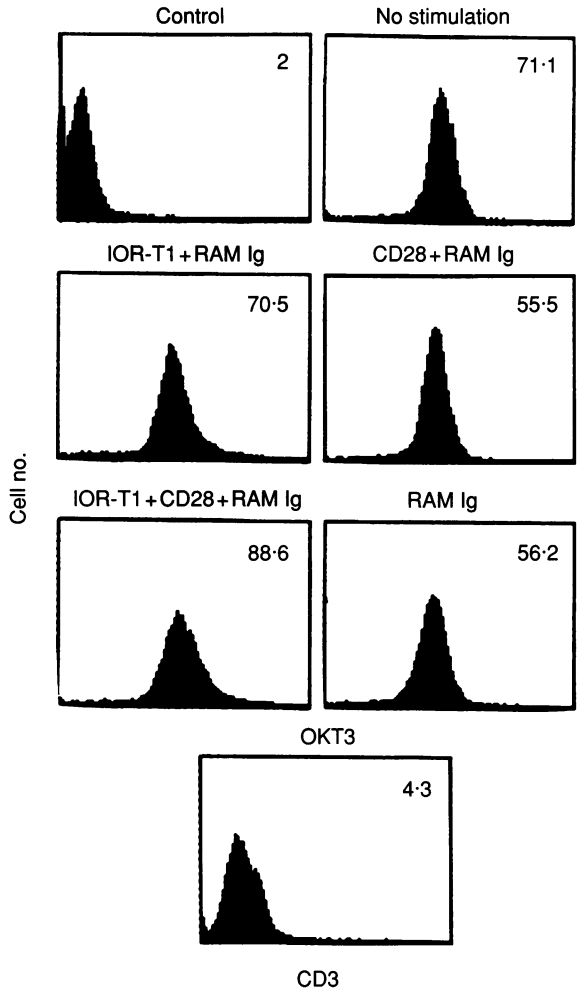

Figure 5. Effect of cross-linking CD6 and CD28 alone or simultaneously on the expression of the TCR/CD3 complex. Purified T cells were stimulated with or without the appropriate mAb where indicated in the presence or absence of RAM immunoglobulin for 1 hr at 37°. The cells were washed and the expression of the TCR/CD3 complex was analysed using flow cytometry with a PE-conjugated anti-CD3 mAb. An unrelated mAb conjugated with PE was used as control. The mean fluorescence intensity of each histogram is shown in the upper right corner.

Effect of cyclosporin A on T-cell proliferation induced by co-ligation of anti-CD6 and anti-CD28

To characterize further the signalling pathways during simultaneous cross-linking of anti-CD6 and anti-CD28 mAb, the effect of the immunosuppressive drug, CsA, on T-cell proliferation induced through these two cell surface molecules was examined. The results in Fig. 6 showed that T-cell proliferation induced through CD6/CD28 ligation was only partially blocked by 0·5 μg/ml CsA (42·8% inhibition) and the inhibition was increased slightly with a higher concentration of CsA (1 μg/ml). At similar concentrations of CsA, T-cell proliferation mediated through the cross-linking of IOR-T1 or OKT3 alone in the presence of TPA, or IOR-T1/OKT3 mAb was completely inhibited (98, 92 and 97%, respectively). In sharp contrast, the T-cell proliferation induced by the cross-linking of anti-CD28 mAb in the presence of TPA was completely resistant to CsA, which is in line with previous reports.[3,26] The concentrations of CsA used were not toxic over the course of the experiments. Taken together these results strongly suggest that the signalling pathways between CD6 and CD28 are distinct.

PKC inhibitors block T-cell proliferation induced by cross-linking of CD6 and CD28

The signalling pathways in CD6/CD28-induced T-cell proliferation were further examined using two PKC-specific inhibitors, H-7 and GF 109203X, the latter being a staurosporine analogue but with higher selectivity for PKC.[27] GF 109203X and H-7 were added to T cells in concentrations ranging from 0·12 to 0·5 μM and from 12·5 to 50 μM, respectively. As shown in Fig. 7, T-cell proliferation induced by cross-linking IOR-T1 and anti-CD28 mAb was inhibited by both PKC inhibitors in a dose-dependent manner. Surprisingly, IOR-T1/OKT3-mediated T-cell proliferation was extremely sensitive to the PKC inhibitors, requiring very low concentration of inhibitors for complete inhibition. In contrast, much higher concentrations of the PKC inhibitors were needed to block CD28/OKT3- and OKT3/TPA-induced T-cell proliferation efficiently. Both PKC inhibitors have similar differential concentration-dependent inhibitory effects on CD6/OKT3-and CD28/OKT3-induced T-cell proliferation (Fig. 7) suggesting that PKC plays a major role in T-cell activation and proliferation.

DISCUSSION

In the present report we showed that peripheral resting T cells can be activated to proliferate by cross-linking mAb to CD6 and CD28 in the absence of TCR/CD3 occupancy. Simultaneous cross-linking of the mAb to the two cell surface molecules is crucial as cross-linking of anti-CD6 or anti-CD28 mAb alone, or when the mAb are in their soluble form, has no effect on T-cell proliferation. We also found that the T cells expressed IL-2 receptors (CD25) and an increase in IL-2 mRNA level following the activation by cross-linking mAb to CD6 and CD28. Both the CD25 and IL-2 mRNA expression is similar to that observed in T cells induced to proliferate with anti-CD6 or anti-CD28 in the presence of TPA as reported previously.[14,19,20] Our present results also suggest that T-cell proliferation induced by cross-linking CD6/CD28 is via an IL-2-dependent autocrine pathway which is similar to cell proliferation mediated through the TCR. We also found that the expression of the TCR/CD3 complex was unaffected during the cross-linking of anti-CD6 and anti-CD28, thus ruling out the possible activation of the cells through the TCR/CD3 complex. The results also suggest that both CD6 and CD28 are not physically associated with the TCR/CD3 complex which has been previously demonstrated with CD4.[25]

The signal transduction mechanism of the CD28 cell surface molecule has been studied in great detail compared to CD6, and that T-cell proliferation mediated through CD28 is completely resistant to the immunosuppressive compounds, CsA and FK506.[3,26] These findings suggest that CD28 is coupled to an intracellular pathway distinct from the TCR/CD3 complex[3,15,16] and evidence supporting this came from various

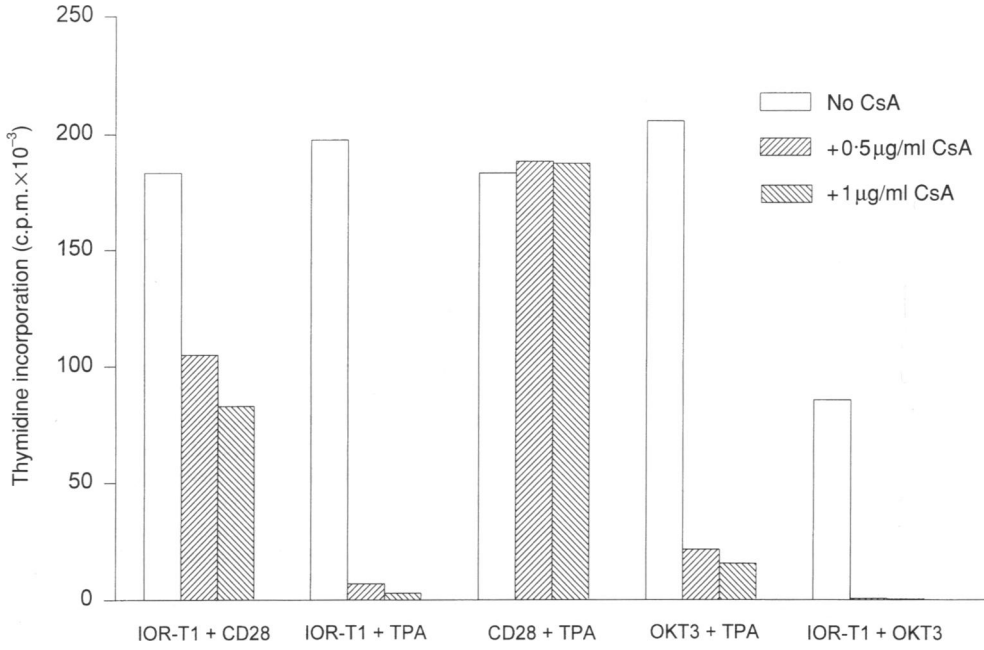

Figure 6. Effect of CsA on CD6- and CD28-mediated T-cell proliferation. Purified T lymphocytes (1×10^5/well) were stimulated with IOR-T1, anti-CD28 mAb, OKT3 and TPA, either alone or in combination where indicated in RAM immunoglobulin-coated 96-well plates in the presence or absence of CsA (0·5 or 1 μg/ml). Cell proliferation was determined in triplicate cultures after 72 hr by [^{3}H]thymidine incorporation, and the results are expressed as the mean counts per minute (c.p.m.) of triplicate cultures. The standard deviations of each mean value were within 15%. This is a representative experiment of three experiments.

reports showing the involvement of PI3-kinase during CD28 co-stimulation.[28,29] It is still not clear if there are important quantitative and/or qualitative differences in the activation of PI3-kinase by CD28 and the TCR or that the co-stimulatory ability of CD28 resides in its capacity to activate signalling pathways other than PI3-kinase.[30] CD28 activation also results in the induction of tyrosine phosphorylation of substrates which appears to be a subset of those obtained after T-cell stimulation through TCR/CD3 complex.[20,24] Similar to CD28, PTK is also involved in the CD6 signalling mechanism during co-stimulation, although the pattern of tyrosine-phosphorylated substrates after CD6/TPA stimulation appears to be similar to those seen after CD3 cross-linking.[14]

To understand further the mechanisms of CD6/CD28-mediated T-cell proliferation, the effects of the immunosuppressive drug, CsA on T-cell proliferation mediated through CD6/CD28 was investigated. CsA was used because it inhibits the activation of the IL-2 gene transcription by blocking the Ca^{2+}-dependent phosphatase, calcineurin[31] and has no effect on CD28 co-stimulation.[3,26] Our results showed that T-cell proliferation mediated through the cross-linking of CD6 and CD28 was only partially inhibited by CsA, whereas cell proliferation induced by CD6 in the presence of anti-CD3 or TPA was completely blocked. In sharp contrast, cross-linking CD28 in the presence of TPA was unaffected by CsA. Although these results suggest that the signalling mechanisms coupled to CD6 and CD28 receptors are distinct, further studies using PKC inhibitors suggested otherwise. We found that T-cell proliferation induced by the cross-linking of CD6 and CD28 mAb together or on their own in the presence of TPA or anti-CD3 were effectively blocked dose-dependently by two PKC inhibitors, GF 109203X and H-7. These findings suggest that the early signals associated with CD28 and CD6 may be similar, for instance PKC activation, but then diverge out into very distinct pathways to regulate different processes associated with cell proliferation. It is surprising that CD6/CD28-induced T-cell proliferation involved PKC activation because both CD6 and CD28 alone cannot induce T-cell proliferation unless in the presence of TPA, which is presumably required to activate PKC. One possible explanation is that both CD6 and CD28 may activate specifically different isoforms of PKC, both isoforms of which are crucial for the activation of cell proliferation and can be activated by TPA. It would be interesting to examine and compare the different PKC isoforms activated during T-cell proliferation mediated by these antigens alone or together.

The physiological significance of this CD6/CD28 antigen-independent pathway of T-cell activation is not clear and similar observations have also been reported with CD5 and CD28.[32] So far, little is known about the role of CD6 in regulating the immune response, whereas the interaction between CD28 on T cells and B7/BB1 on APC has been shown to provide co-stimulus to the T cells and to rescue T cells from activation-induced cell death.[33] Although the CD6 ligand has been found in lymphoid tissues, including the skin, lymph nodes and thymus, it is still unclear whether CD6 plays any role in modulating T-cell activation in lymphoid tissues and T-cell maturation in the thymus. Incidentally, CD28 is expressed in the CD3/TCR-negative thymocytes and the T-cell activation pathway in these cells appears to be functional via CD28 activation.[34] It would be interesting to see if these thymocytes also express CD6. It remains to be determined whether thymic epithelial cells expresses both CD6 and CD28 ligands and whether cells having both CD6 and CD28 ligands

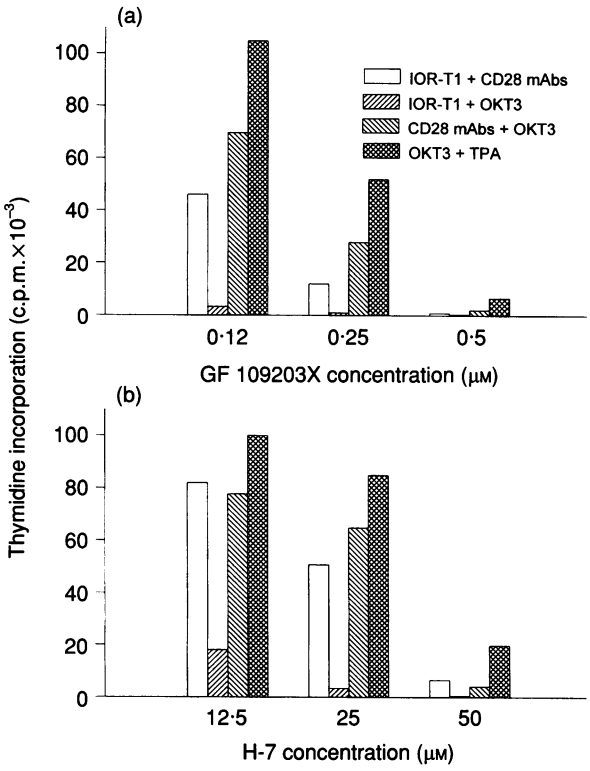

Figure 7. Effect of PKC inhibitors on T-cell proliferation induced by cross-linking of anti-CD6 and anti-CD28 mAb. Purified T lymphocytes (1×10^5/well) were stimulated with IOR-T1, anti-CD28 mAb, OKT3 or TPA alone or in the indicated combination on RAM immunoglobulin-coated 96-well plates. Cells were preincubated with GF 109203X or H-7 at the indicated concentrations for 15 min prior to stimulation. Proliferation was determined after 72 hr by [^{3}H]thymidine incorporation and is expressed as percentage of the response observed in the absence of inhibitors (control). The results are the means from triplicate samples from one representative experiment out of three performed. Cell proliferation in counts per minute (c.p.m.) without inhibitors are: control, 73±9; CD6+CD28, 106 121±13 480; CD6+OKT3, 20 951±3405; CD28+OKT3, 142 659±1520 and OKT3+TPA, 88 852±10 633.

can activate T cells. Further studies are clearly required to establish the significance of this antigen-independent pathway of T-cell activation.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Agency SAREC (to M.J.), The Swedish Medical Association and Karolinska Institute (to S.C.C.) and Swedish Institute (to L.M.O.).

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