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Ligation of the CD5 or CD28 molecules on resting human T cells induces expression of the early activation antigen CD69 by a calcium- and tyrosine kinase-dependent mechanism

P. VANDENBERGHE, J. VERWILGHEN, F. VAN VAECK & J. L. CEUPPENS *Division of Clinical Immunology, Department of Internal Medicine and Pathophysiology, University of Leuven, Belgium*

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SUMMARY

The CD5 and CD28 molecules on T lymphocytes can each exert an accessory role in T-cell activation. Ligands for CD5 and CD28 have been identified as CD72 and B7/BB1 respectively. The function of, and the signal transduction pathways coupled to CD28 have been the subject of extensive studies. In contrast, it is still debated whether CD5 functions as a receptor which directly transduces an independent signal to the T cell. In this paper, it is reported that culture of purified T cells in the presence of either immobilized anti-CD5 monoclonal antibody (mAb) (OKT1, Leu-1 or 10.2) or cross-linked anti-CD28 (9.3) mAb (but not of anti-LFA-1α, anti-LFA-1β, or anti-CD7) induces expression of CD69, an early activation marker, in the absence of other activating stimuli. CD69 expression was consistently detectable after 3–24 hr on 20–50% of T cells, within both the CD4 and CD8 subsets. $CD45RO^-$ $CD45RA^+$ naive T cells were more responsive than $CD45RO^+$ $CD45RA^-$ memory T cells. In the presence of recombinant (r) interleukin-2 (IL-2), anti-CD5- or anti-CD28-induced CD69 expression was further up-regulated, more sustained and, as previously shown, succeeded by IL-2 responsiveness. Simultaneous cross-linking of both CD5 and CD28 enhanced CD69 expression above the levels obtained with optimal amounts of both ligands separately. In the presence of a submitogenic dose of the protein kinase C (PKC) activating agent phorbol 12-myristate 13-acetate (PMA), co-stimulation with anti-CD5 or anti-CD28 increased CD69 expression above that induced by PMA alone. Cross-linking of CD5 or CD28 induces an early rise of cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) and both this rise and CD69 expression were inhibited by chelation of extracellular Ca^{2+} with ethyleneglycol-*bis*-(2-aminoethyl)-tetraacetate (EGTA). Pretreatment of the cells with the tyrosine kinase inhibitor herbimycin A also blocked CD69 expression. The data thus further corroborate a role for CD28 and for CD5 as receptors able to transduce signals to T cells in an antigen-independent fashion. Moreover it is demonstrated that influx of Ca^{2+} and tyrosine kinase activity are involved in the signal transduction pathways of both receptors.

INTRODUCTION

The minimal requirement for a T-lymphocyte response to antigen is binding of the processed antigen and of the major histocompatibility complex (MHC) molecule on an antigen-presenting cell (APC) to the T-cell receptor (TcR)/CD3 complex on a clonally distributed T lymphocyte. The outcome of TcR/CD3 stimulation, T-cell proliferation versus T-cell anergy, critically depends on the concomitant delivery of co-stimulatory 'accessory' signals by the APC.[1] Among others, the CD28 antigen on mature thymocytes and T lymphocytes has received much attention as a receptor for such accessory signals,[2–9] (for review see ref. 2). A natural ligand for CD28 has been identified as B7/BB1, expressed on activated B cells, activated monocytes and thymic interlobar tissue,[10–12] and interaction between CD28 and B7/BB1 delivers the accessory signal.[8–9] Another potential receptor for accessory signals is CD5, a natural ligand which was recently identified as CD72,[13] expressed on pre-B cells and mature B cells. However, for CD5 it is incompletely resolved whether ligation of this molecule merely stabilizes or anchors the binding of mAb to CD3, or whether it functions as a receptor which directly transduces an independent signal to the T cell

Abbreviations: APC, antigen-presenting cell; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; EGTA, ethyleneglycol-*bis*-(2-aminoethyl)-tetraacetate; FCS, foetal calf serum; GAM IgG, goat anti-mouse immunoglobulin G; HBSS, Hanks' balanced salt solution; IL-2R, interleukin-2 receptor; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; rIL-2, recombinant interleukin-2.

Correspondence: Dr J. L. Ceuppens, Clinical Immunology, University Hospital St. Raphaël, Provisorium II, Kapucijnenvoer 33, B-3000 Leuven, Belgium.

after binding to its presumed ligand CD72. Several groups, including our own, have demonstrated that CD5 can function as a receptor for co-stimulatory signals in the presence of TcR/CD3 stimulation, protein kinase C (PKC)-activating phorbol esters of exogenous interleukin-2 (IL-2),[4,5,14–16] and these effects in fact bear similarity to those of CD28 ligation. Cross-linking of both CD28 or CD5 results in an increase of cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in T lymphocytes.[17,18] No direct evidence for PKC activation after CD28 ligation has been reported, but recently it was found that a 100,000 MW substrate is phosphorylated by a protein tyrosine kinase after CD28 ligation.[19] As to CD5, one particular anti-CD5 mAb (Cris1) induces PKC and protein tyrosine kinase activity, the latter with a substrate pattern that differs from that induced after triggering of the CD3/TcR complex.[20] Because the latter mAb has the unique property of being mitogenic by itself, it is unclear to what extent this effect can be generalized to CD5 ligation in general.

In the present study, the problem of signal transduction through CD5 and CD28 was approached in a different way, by analysing the expression of CD69. CD69 is a disulphide-linked dimeric structure (MW 28,000–32,000), which was described as MLR3,[21,22] activation inducer molecule (AIM),[23,24] early activation antigen (EA1)[25–27] and Leu-23.[28–30] CD69 is not present on resting T lymphocytes, but is the earliest membrane activation marker induced after stimulation with anti-CD3, mitogenic lectins or PMA. Risso and co-workers recently reported that immobilized anti-CD28 induces CD69 expression.[31] It was found that treatment of purified human T lymphocytes with immobilized anti-CD5 mAb also induces CD69 expression, thus providing more evidence for the similar functions of CD5 and CD28. Moreover it was found that this occurred for both CD5 and CD28 ligation by a signalling pathway requiring extracellular Ca^{2+} influx and activation of a protein tyrosine kinase.

MATERIALS AND METHODS

Cells

Blood donors for these experiments were healthy male or female volunteers, aged 20–50 years. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradients (density 1·077 kg/l). After three washes in Hanks' balanced salt solution (HBSS) (Gibco, Paisley, U.K.) containing 1% foetal calf serum (FCS) (Gibco), the cells were resuspended in RPMI-1640 (Gibco) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS. After removing monocytes by cold agglutination,[32] T cells were further purified by using complement-fixing monoclonal antibody (mAb) and Lymphokwik-T (One Lambda Inc., Los Angeles, CA). Lymphokwik-T was added to 30×10^6 cells in 200 μl of medium, followed by incubation for 1 hr at 37°. After three washes, the cells were incubated for 30 min at 4° with a mixture of complement fixing anti-natural killer (NK) cell and anti-monocyte mAb of the IgM subclass (anti-CD16, anti-CD56, anti-CD57 and anti-CD14), and washed once. Then 0·8 ml of Lymphokwik-T reagent was added again for 1 hr, followed by three washes. This procedure yielded a cell population containing more than 97% T cells ($CD3^+$), 65–70% $CD4^+$ cells, 30–35% $CD8^+$ cells, and less than 0·5% monocytes ($CD14^+$), as determined by flow cytometry. Cells were cultured in RPMI-1640 medium (Gibco), supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% serum. Identical results were obtained with normal human serum, FCS or foetal bovine serum (FBS). Stimulants added to the cultures were phorbol 12-myristate 13-acetate (PMA) (Calbiochem, La Jolla, CA), recombinant (r)IL-2 (Janssen Life Sciences, Beerse, Belgium) or the indicated mAb. In order to block tyrosine kinase activity, cells were preincubated with herbimycin A[33,34] overnight, before addition of the stimulants. Herbimycin A (Gibco BRL, Gaithersburg, MD) was prepared as a 1·75 mM stock solution in DMSO, and used at a final concentration of 1 μM which did not affect cell viability as assessed by trypan blue exclusion.

Monoclonal antibodies used for cell culture

The following mAb were used in cell culture experiments: anti-CD3 mAb anti-Leu-4 (IgG1); anti-CD28 mAb 9.3 (IgG2a); anti-CD5 mAb 10.2 (IgG2a), anti-Leu-1 (IgG2a) or OKT1 (IgG1); anti-LFA-1α mAb IOT16 (IgG1); anti-LFA-1β mAb IOT18 (IgG1) and anti-CD7 3A1 (IgG1). Purified mAb 10.2 and 9.3 were kindly donated by Dr P. J. Martin (Fred Hutchinson Cancer Research Center, Seattle, WA). Monoclonal antibody 3A1 ascites was a gift from Dr A. Fauci (NIH, Bethesda, MD). Anti-Leu-1 and OKT1 were purified from hybridoma (ATCC, Rockville, MD) supernatant by affinity chromatography on protein G (Pharmacia). Purified anti-Leu-4 was purchased from Becton Dickinson (Erembodegem, Belgium). Purified IOT16 and IOT18 were purchased from Immunotech (Marseille, France). Monoclonal antibodies were cross-linked through addition of soluble goat anti-mouse immunoglobulin G (GAM IgG) (Tago, Burlingame, CA). Immobilization of mAb was obtained by direct coating of culture wells with mAb or through absorption of mAb to culture wells coated with GAM IgG as described previously.[15] All antibodies used in cultures were extensively dialysed before use.

Single- and dual-colour immunofluorescence analysis

Cells were incubated with the relevant fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAb for 30 min at 4° in a total volume of 100 μl. Controls included FITC- or PE-conjugated mouse IgG of the relevant subclasses. CD69 expression was examined with FITC- or PE-conjugated mAb anti-Leu-23 (a gift from F. Nauwelaers, Becton Dickinson). In some experiments, double staining with PE- or FITC-conjugated anti-CD4 mAb anti-Leu-3a, anti-CD8 mAb anti-Leu-2a, anti-CD45RO mAb UCHL-1 (all from Becton Dickinson), anti-CD45RA mAb 2H4 or anti-CD29 (integrin β1) mAb 4B4 (both from Coulter, Hialeah, FL) along with FITC- or PE-conjugated anti-Leu-23 was performed. After three washes, cells were fixed in 1% paraformaldehyde and analysed on a FACScan (Becton Dickinson). Forward and right-angle scatter gates were set to include lymphocytes and lymphoblasts.

Flow cytometric measurement of $[Ca^{2+}]_i$ with fluo-3

The calcium-sensitive fluorochrome fluo-3, and pluronic F-127 were purchased from Becton Dickinson. 10^7 T cells/ml were loaded for 20 min with the calcium-sensitive fluorochrome fluo-3 (4 μM in HBSS) and with pluronic F-127 (75 μg/ml) as previously described.[35] Next, cells were diluted to 2×10^6 in HBSS with 1% FCS and incubated for 40 min. After washing, cells were resuspended at 1×10^6/ml in saline with NaCl 137 mM, KCl 5 mM, Na_2HPO_4 1 mM, glucose 5 mM, $CaCl_2$ 1 mM, $MgCl_2$

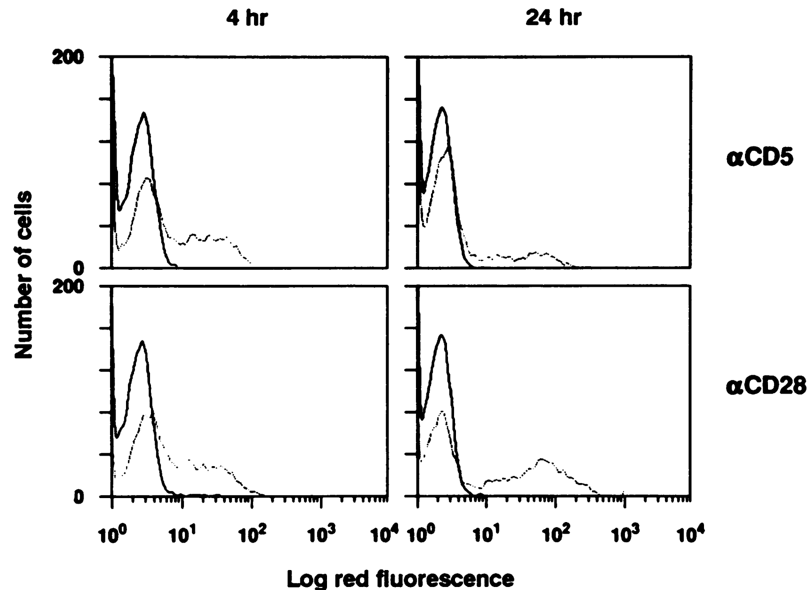

Figure 1. Immobilized anti-CD5 mAb and anti-CD28 mAb induce the expression of CD69 in resting T cells. T cells were incubated with mAb of the indicated specificity for 1 hr at 4°, followed by culture on plates coated with GAM IgG for 4 or 24 hr. Cells were stained with PE-conjugated anti-CD69 mAb Leu-23 and analysed by flow cytometry as described in Materials and Methods. Overlay histograms of CD69 expression by unstimulated (——) and stimulated (······) cells are shown. Anti-CD5 mAb 10.2 was used at 100 ng/ml, anti-CD28 mAb 9.3 at 500 ng/ml. These optimal mAb concentrations had been determined in preliminary experiments (not shown). Similar results were obtained with anti-CD5 mAb OKT1 and anti-Leu-1.

0·5 mM, HEPES 10 mM and FCS 1%, pH 7·4 at 37°. Cellular fluorescence of fluo-3 was measured by argon laser excitation at 488 nm and detection at 525 nm on a linear scale, with forward and right-angle scatter gates on the lymphocyte population. Monoclonal antibodies were added in a final concentration of 10 μg/ml at time −6 min, followed by cross-linking with GAM IgG (40 μg/ml) at time 0. When used, ethyleneglycol-*bis*-(2-aminoethyl) (EGTA) (Fluka, Buchs, Switzerland) was added in a final concentration of 1·0 mM, together with the primary antibody at time −6 min.

RESULTS

Immobilized anti-CD5 and cross-linked anti-CD28 mAb induce CD69 expression

Purified T cells were stimulated for 4–24 hr with immobilized anti-CD5 or anti-CD28 mAb. As shown in Fig. 1, both immobilized anti-CD5 (mAb 10.2) or anti-CD28 (mAb 9.3) induced the expression of CD69 on the T-cell surface. A peak response after CD5 stimulation generally occurred after 4–6 hr of stimulation, whereas the maximal response to CD28 was more delayed until 24 hr of stimulation. After 48 hr, CD69 expression on T cells had returned to background level (not shown). Two out of two other anti-CD5 mAb tested, anti-Leu-1 and OKT1, similarly induced CD69 on resting T cells. Immobilized or cross-linked mAb identifying CD7 (3A1), LFA-1α (IOT16) and LFA-1β (IOT18) did not induce CD69 expression under similar conditions (Table 1). At any time, the surface expression of CD69 after CD5 or CD28 ligation was dimmer and on a lower percentage of cells than that induced with optimal amounts of immobilized anti-CD3 or PMA (not shown). T cells stimulated with immobilized anti-CD5 mAb or anti-CD28 mAb did not express IL-2R (p55) after 24 or 48 hr.

Table 1. Anti-CD7, anti-CD11a and anti-CD18 mAb do not induce CD69 expression in resting T cells*

mAb	Specificity†	% of T cells expressing CD69‡
Medium	—	2
9.3	CD28	30
3A1	CD7	2
IOT18	CD18	3
IOT16	CD11a	3

* T cells (2×10^6/ml) were incubated in 24-well plates, coated with GAM IgG.

† mAb of the indicated specificities were added in a final concentration of 500 ng/ml.

‡ CD69 expression was measured by direct immunofluorescence after 4 hr.

In the previous experiments, anti-CD5 or anti-CD28 were presented in immobilized form, in order to mimic the physiological situation of binding to cell-bound ligands.[8,9,13] The CD69 response to soluble, cross-linked and immobilized mAb was also examined. Intact bivalent anti-CD28 mAb was inactive. It was possible to induce CD69 through cross-linking of the CD28 receptor with mAb 9.3 (1–10 μg/ml) followed by soluble GAM IgG, although the response was less pronounced than with anti-CD28 mAb presented on a solid support. In contrast, CD69 induction through CD5 ligation strictly required presentation of the anti-CD5 mAb on a solid support, either through direct coating of culture plates with purified mAb or through absorption of the mAb on culture plates coated with GAM IgG (not shown). Thus, CD69 expression through triggering of CD5 or

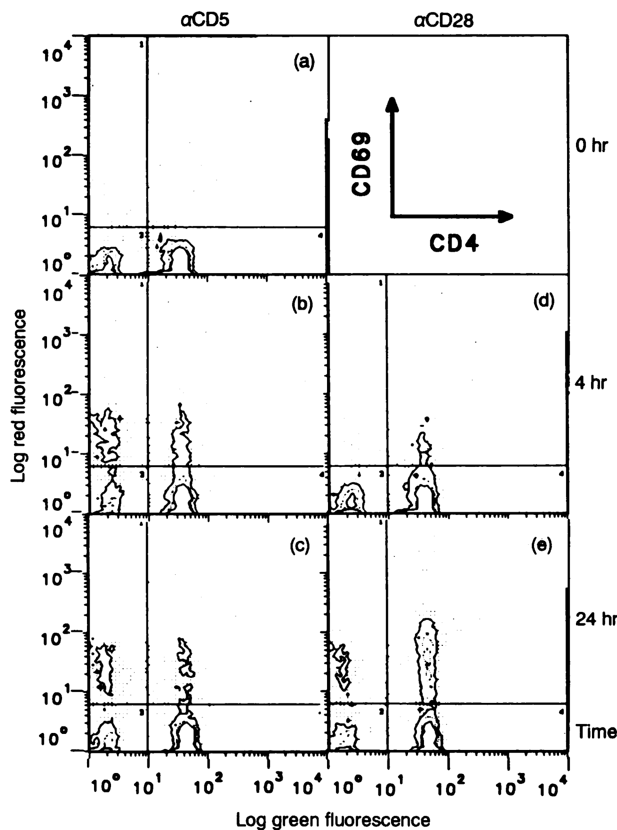

Figure 2. Both CD4$^+$ and CD4$^-$ T-cell subsets express CD69 after stimulation with immobilized anti-CD5 or anti-CD28 mAb. T cells were cultured in the absence (a) or presence of immobilized anti-CD5 mAb 10.2 (100 ng/ml) (b, c) or immobilized anti-CD28 mAb 9.3 (500 ng/ml) (d, e). After 4 (b, d) or 24 hr (c, e), cells were recovered and stained with FITC-conjugated anti-CD4 mAb Leu-3a and with PE-conjugated anti-CD69 mAb Leu-23. Cellular staining was analysed by dual-colour immunofluorescence.

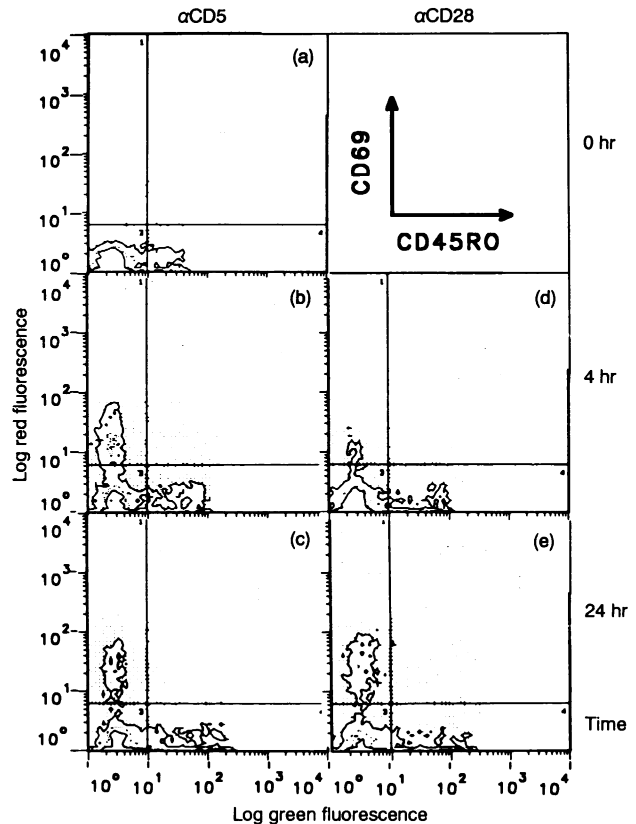

Figure 3. Expression of CD69 after stimulation with immobilized anti-CD5 or anti-CD28 mAb occurs predominantly but not exclusively in the naive T-cell subset. T cells were cultured in the absence (a) or presence of immobilized anti-CD5 mAb 10.2 (100 ng/ml) (b, c) or immobilized anti-CD28 mAb 9.3 (500 ng/ml) (d, e). After 4 (b, d) or 24 hr (c, e), cells were recovered and stained with FITC-conjugated anti-CD45RO mAb UCHL1 and with PE-conjugated anti-CD69 mAb Leu-23. Cellular staining was analysed by dual-colour immunofluorescence.

CD28 occurs only after multivalent ligation of these receptors, as it occurs after binding to cell-bound ligands.

The CD69 response to immobilized anti-CD5 and anti-CD28 mAb occurs predominantly in the naive T-cell subset

CD69 expression in the response to CD28 and CD5 is restricted to less than 50% of the cells (Fig. 1). As this might reflect the differential responsiveness of distinct T-cell subpopulations, CD69 expression was investigated with dual-colour flow cytometry, using FITC-conjugated mAb distinguishing CD4$^+$ helper versus CD8$^+$ suppressor–cytotoxic lymphocyte subsets (anti-CD4 mAb anti-Leu-3a, anti-CD8 mAb anti-Leu-2a) or naive versus memory lymphocyte subsets [anti-CD45RO mAb UCHL1, anti-CD45RA mAb 2H4, or anti-integrine β_1 (CD29) mAb 4B4], along with PE-conjugated anti-Leu-23. The CD69 response was observed in both CD4$^+$ and CD4$^-$ lymphocyte subsets (Fig. 2), or in CD8$^+$ and CD8$^-$ subsets (not shown). This indicates that both helper (CD4$^+$ CD8$^-$) and suppressor–cytotoxic (CD4$^-$ CD8$^+$) participate in the CD69 response. CD69 was however preferentially expressed among CD45RO$^-$ naive T cells compared to CD45RO$^+$ memory T cells (Fig. 3). Still, significant percentages of CD45RO$^+$ cells (up to 20–25%) could express CD69 at 4–24 hr after ligation of CD28 or CD5. Similar results were also obtained when 2H4 or 4B4 mAb were used to distinguish naive and memory cells (data not shown).

Effect of combined CD5 and CD28 ligation on CD69 expression

CD5 and CD28 ligation recruited only a low proportion of cells to express CD69, even if mAb concentrations were further increased. However, with the combination of optimal concentrations of immobilized anti-CD5 and anti-CD28 in the same cultures, CD69 was strongly up-regulated, with an increase in both the staining intensity and in the number of positive cells (Fig. 4). Again expression was more clearly observed on naive T cells than on memory T cells (not shown).

Anti-CD28- and anti-CD5-induced expression of CD69 is enhanced in the presence of exogenous rIL-2

When T cells are stimulated with immobilized anti-CD28 mAb or anti-CD5 mAb in the absence of rIL-2, they do not express IL-2R (p55), as analysed by flow cytometry. As already demonstrated, the same stimulation conditions in the presence of exogenous rIL-2 paradoxically induce a low percentage of cells to express the p55 IL-2R and to proliferate.[6,16,17] This

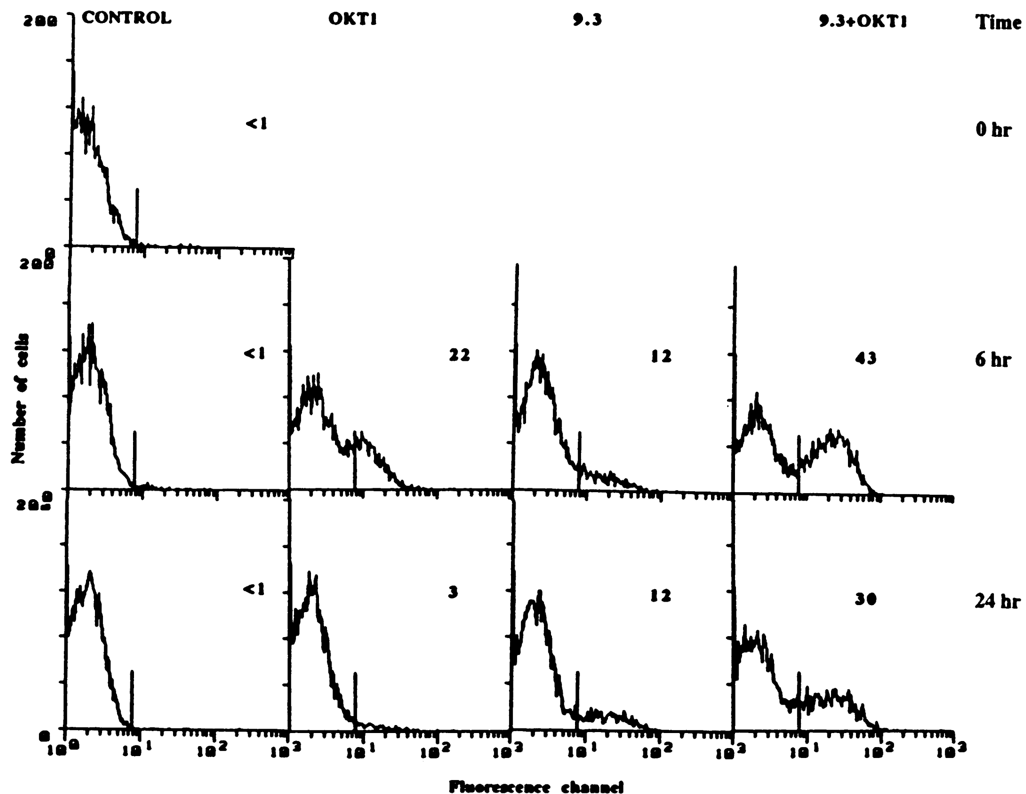

Figure 4. Induction of CD69 expression by the combination of immobilized anti-CD5 and anti-CD28. T cells were incubated on plates coated with GAM IgG in the presence or absence of OKT1 (1 μg/ml) and of mAb 9.3 (100 ng/ml). At the indicated time-points, cells were stained with FITC-conjugated anti-CD69 and analysed by flow cytometry. The percentage of cells above the marker point is shown in the right upper corner.

proliferation proceeds by an IL-2-dependent mechanism, since it was sensitive to inhibition with anti-IL-2R (p55) mAb anti-TAC.[6,16] It is speculated that these effects were due to binding of rIL-2 to p55 IL-2R, expressed below the detection threshold of the flow cytometer used, or to p75 IL-2R. Because of the above effect of exogenous IL-2 on IL-2R expression and cell proliferation, the effect of rIL-2 on the induction of CD69 was examined after CD5 or CD28 ligation (Fig. 5a). Recombinant rIL-2 (10–100 U/ml) by itself did not induce significant CD69 expression in resting T cells. In the presence of rIL-2 (100 U/ml) however, immobilized anti-CD5 and anti-CD28 mAb induced CD69 more efficiently, with higher percentages of positive T cells and with a more prolonged time-course (Fig. 5a). This could be observed as early as 4 hr after the start of stimulation. Recombinant rIL-2 at 10 U/ml did not affect CD5- or CD28-induced CD69 expression.

Ligation of CD5 or CD28 enhances PMA-induced CD69 induction

PMA and other PKC-activating agents are strong inducers of CD69.[24,25] CD69 expression was therefore examined by T cells stimulated with anti-CD5 or anti-CD28 mAb together with PMA. When PMA was used in a concentration (0·5 ng/ml) which suboptimally induces CD69 expression, immobilized anti-CD5 or anti-CD28 augmented both the number of positive cells (Fig. 5b) and the cellular CD69 brightness (not shown). With PMA 1 ng/ml, virtually all cells expressed CD69 after 4 hr of stimulation. Under these conditions, immobilized anti-CD28 and anti-CD5 exerted a moderate effect on cellular CD69 brightness but not on the percentage of CD69$^+$ cells (data not shown).

An increase of $[Ca^{2+}]_i$ is required for CD28- and CD5-triggered CD69 induction

The ability to increase sustainedly $[Ca^{2+}]_i$ appears to be an important co-variable for CD69 expression when T cells are stimulated with anti-CD3.[29] Cross-linking of CD5 as well of CD28 results in an increase in $[Ca^{2+}]_i$.[17,18] The role of $[Ca^{2+}]_i$ changes induced by CD5 or CD28 cross-linking was therefore investigated. Purified T cells were stimulated with immobilized anti-CD5 or anti-CD28 mAb in the presence or absence of EGTA, a chelator of extracellular calcium, and both the $[Ca^{2+}]_i$ and the CD69 response were measured (Fig. 6). Calcium depletion of the extracellular melieu with EGTA completely inhibited the $[Ca^{2+}]_i$ increase and abolished CD69 expression after CD5 or CD28 stimulation. This indicates that a rise of $[Ca^{2+}]_i$ is involved in coupling the ligation of CD5 and/or CD28 to subsequent CD69 expression.

Tyrosine kinase activity is required for CD69 induction

Recently it was demonstrated that triggering of either CD5 or CD28 induces tyrosine phosphorylation of intracellular substrates.[19,20] To examine the potential involvement of a protein

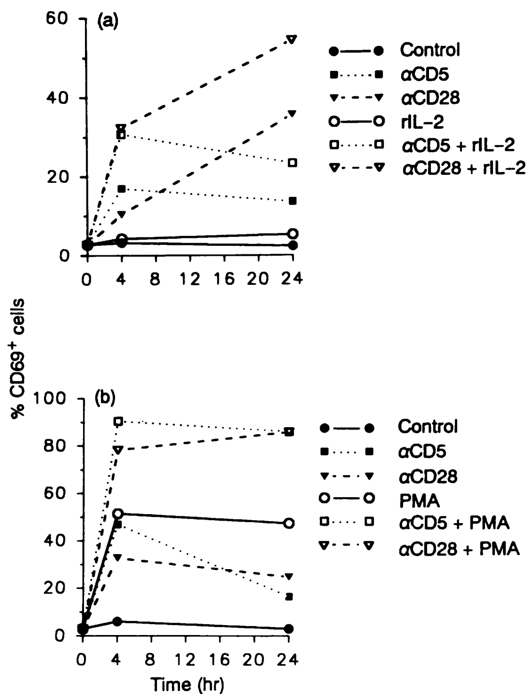

Figure 5. CD5 or CD28 induced expression of CD69 in the presence of rIL-2 or of PMA. (a) T cells were stimulated with immobilized anti-CD5 mAb 10.2 (100 ng/ml) or anti-CD28 mAb 9.3 (500 ng/ml) as indicated, in the presence or absence of exogenous rIL-2 (100 U/ml). After 4 or 24 hr, cells were recovered and CD69 expression was determined as described in Fig. 1. The percentage CD69$^+$ cells after 4 and 24 hr of stimulation is shown. (b) T cells were cultured with immobilized anti-CD5 mAb 10.2 (100 ng/ml) or anti-CD28 mAb 9.3 (500 ng/ml) in the presence or absence of PMA (0·5 ng/ml), as indicated. After 4 or 24 hr, cellular CD69 expression was determined as described in Fig. 1. The percentage of CD69$^+$ cells after 4 and 24 hr of culture is shown.

tyrosine kinase in CD5- or CD28-induced expression of CD69, herbimycin A was used, an inhibitor of *sarc*-family tyrosine kinases.[33–34] Before stimulation with immobilized anti-CD5 or anti-CD28, T cells were preincubated with herbimycin A (1 μM) overnight, as described previously.[34] As can be seen in Table 2, herbimycin A almost completely blocked CD69 expression induced by these mAb either alone or in combination. In contrast, CD69 expression induced by PMA (10 ng/ml) was not blocked (Table 2).

DISCUSSION

The activation marker CD69 is expressed rapidly on T cells following stimulation with mitogenic stimuli (anti-CD3, mitogenic pairs of anti-CD2, mitogenic lectins) or PKC-activating agents.[21–26,29] In this paper, it is reported that non-mitogenic T-cell stimulation with immobilized anti-CD5 or cross-linked anti-CD28 mAb can also induce dim and transient expression of CD69 on a proportion of resting T cells.

Cross-linking of CD5 and CD28 was required for CD69 expression, consistent with the concept that the natural ligands of both receptors are cell surface molecules and not soluble cytokines.[8,9,13] The CD5 receptor required mAb presentation on a solid support, whereas for CD28 soluble cross-linking was sufficient. If presented this way, both anti-CD5 and anti-CD28 mAb have previously been shown to induce IL-2R (p55) expression and functional IL-2 responsiveness in the presence of rIL-2.[6,16] In addition, as shown in Fig. 5, ligation of CD5 or CD28 and rIL-2 act synergistically to increase CD69 expression at 4 and 24 hr. The identical mAb presentation requirements for both CD5 and CD28, and the synergism between IL-2 and CD5 or CD28 cross-linking suggest that expression of CD69 and, more distally, of IL-2R (p55) are somehow coupled through a common signal transduction pathway.

Only a limited number of cells responded to CD5 or CD28 ligation with CD69 expression. The CD69 response was com-

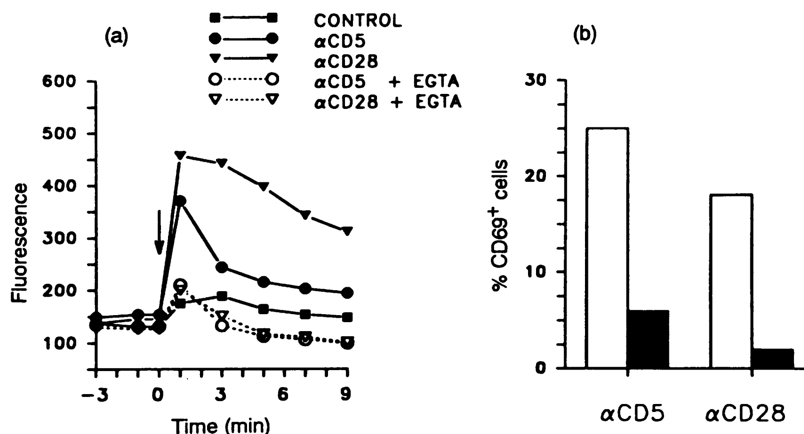

Figure 6. Chelation of extracellular calcium inhibits the increase of $[Ca^{2+}]_i$ and CD69 response after ligation of CD5 and CD28. (a) Fluo-3 loaded T cells were incubated with anti-CD5 or anti-CD28 mAb (10 μg/ml) at time −6 min, in the presence or absence of EGTA 1·0 mM. GAM IgG (40 μg/ml) was added at time 0 (arrow). Cellular fluorescence was sampled at 2-min intervals, acquiring 2000 events at each point. The graph shows mean cellular fluorescence at each time-point, as calculated with FACScan software. (b) T cells were stimulated with immobilized anti-CD5 mAb 10.2 (100 ng/ml) or anti-CD28 mAb 9.3 (500 ng/ml) as described in Materials and Methods in the absence (□) or presence (■) of EGTA 1·0 mM. After 4 hr CD69 expression was determined as in Fig. 1. Percentages of positive cells are shown.

Table 2. Effect of a PTK-inhibitor (herbimycin A) on CD69 expression induced by cross-linking of CD5 or CD28*

	% of $CD45RO^-$ T cells expressing CD69†			
	Donor 1		Donor 2	
Culture addition	Medium	Herbimycin	Medium	Herbimycin
Medium	< 1	< 1	< 1	< 1
Anti-CD5	19	4	21	3
Anti-CD28	14	< 1	52	22
Anti-CD5+anti-CD28	39	6	56	10
PMA (4 hr)	100	100	98	98
PMA (24 hr)	87	89	92	100

* T cells (2×10^6/ml) were incubated in 24-well plates, coated with GAM IgG. mAb 10.2 (100 ng/ml), mAb 9.3 (500 ng/ml), and (or) herbimycin A (1 μM) were added as indicated.

† CD69 expression was measured by direct immunofluorescence after 4 hr (for anti-CD5-stimulated cells) or 24 hr (for anti-CD28 or anti-CD28/CD5-stimulated T cells) of culture. PMA was used at 10 ng/ml and PMA-stimulated cultures were harvested at 4 and 24 hr as indicated. Results are given for $CD45RO^-$ cells. Viability of the T cells always exceeded 98%.

parable in the $CD4^+$ or $CD8^+$ subsets, but more pronounced among $CD45RO^-$ $CD45RA^+$ naive T cells than among $CD45RO^+$ or $4B4^+$ memory T cells. Still, within the naive T-cell subset, marked heterogeneity in CD69 brightness was observed and it therefore remains unclear which other variable(s) control(s) the CD69 response on a cellular level after ligation of CD5 or CD28. Expression of functional IL-2R and of the p55 chain of the IL-2R after binding of CD5 or CD28 in the presence of exogenous rIL-2, similarly occurs in a subpopulation of T cells.[6,16]

Indirect evidence suggest that expression of CD69 is regulated by PKC activation. Indeed, phorbol esters strongly induce CD69. Moreover, induction of CD69 by more physiological stimuli such as anti-CD3 or anti-CD28, is inhibited by H7 and staurosporin.[29,31] Although PKC activation has been demonstrated after ligation of CD5,[20] it has to date not been reported for CD28. To gain better insight into the flow in signalling cascades activated upon ligation of CD5 or CD28, the effect of inhibition of other signal transduction pathways, known to be coupled to these receptors, was examined. After TcR/CD3 triggering, a sustained increase in intracellular calcium has been shown to be an essential requirement for CD69 induction.[29] Cross-linking of CD5 or of CD28 also induces an increase of $[Ca^{2+}]_i$, which was measured with fluo-3. Chelation of extracellular calcium inhibited changes of $[Ca^{2+}]_i$, as well as CD69 induction through CD5 or CD28 ligation. This indicates that a rise of $[Ca^{2+}]_i$ by influx from the extracellular compartment contributes to coupling the ligation of CD5 or CD28 to subsequent CD69 expression. Recently, both ligation of CD5 and of CD28 have been shown to induce activation of a protein tyrosine kinase.[19,20] The experiments reported here with herbimycin A, a tyrosine kinase inhibitor, demonstrate that activation of a tyrosine kinase is also essential in signal transduction leading to CD69 expression. These findings, do not, of course, exclude the possibility that still other signal transduction pathways may couple to these receptors.[20,36]

The induction of the early T-cell activation marker CD69 after ligation of CD5 clearly supports our previous reports on antigen-independent T-cell activation through this receptor.[14-16] CD5 thus seems to be an accessory signal receptor, which can directly transduce signals, rather than merely stabilize or anchor the binding of mAb to CD3.

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