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TRANSIENT EXPRESSION OF THE CD2 CELL SURFACE ANTIGEN AS A SORTABLE MARKER TO MONITOR HIGH FREQUENCY TRANSFECTION OF HUMAN PRIMARY B CELLS[1]

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We have used the T cell surface molecule CD2 gene, expressed from the human cytomegalovirus promoter as a reporter to optimize a transfection system for human primary B cells. The CD2-encoding DNA was transfected into cells by electroporation and transient expression was monitored by flow cytometric analysis. By using our optimal electroporation conditions on activated primary B cells, more than 30% of the resulting viable cells expressed CD2 on the cell surface. Moreover, unactivated primary B cells could also be transfected using this system but subsequent expression of CD2 required cellular activation. Magnetic beads or plastic culture bottles coated with anti-CD2 antibodies have been used to selectively purify transfected cells. The high transfection efficiency combined with the ability to specifically purify transfected cells may allow future studies on specific genes transiently expressed in human primary B cells.

Studies on gene expression using DNA transfection systems are normally difficult in primary human lymphocytes due to low uptake frequencies and low survival rates of treated cells. In recent years the method of electroporation has been used for DNA transfection in a variety of cell types, including various types of primary cells (1, 2). Considering that electroporation is a purely physical mean of allowing DNA entry into cells, as opposed to chemical or biologic methods, it has been suggested that this technique can be optimized to achieve high transfection efficiency in any cell type. The present study was initiated in order to develop an efficient electroporation protocol for primary human B cells. To monitor transient expression, we used a cDNA encoding the cell surface protein CD2 (3) as a reporter gene. This strategy enabled us to estimate transfection efficiency both quantitatively and qualitatively by flow cytometric analysis. Moreover, by cotransfecting the CD2 and CAT genes into primary B cells, we could show that surface expression of CD2, in combination with the use of anti-CD2 antibodies, allowed enrichment of CAT-expressing cells. Hence, in the present study CD2 has successfully been used as both a reporter gene and a sortable marker to specifically purify viable cotransfected human primary B cells.

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MATERIALS AND METHODS

Materials. PDB[3] (Sigma Chemical Co., St. Louis, MO) and ionomycin were dissolved in DMSO at 2 and 5 mg/ml, respectively, and stored at −70°C. BCGF was purchased from Cellular Products Inc. (Buffalo, NY). IL-2 was obtained from Cetus Corp. (Emeryville, CA). Anti-CD2 and anti-CD19 immunomagnetic beads and magnetic separator were purchased from Dynal, Oslo, Norway. OKT11 antibodies (4) were purified from ascites (hybridoma was obtained from the American Type Culture Collection, Rockville, MD). Fluorescein-conjugated anti-Leu 5b antibodies were purchased from Becton Dickinson (Mountain View, CA). FCS was purchased from Sera Lab (Sussex, England). RPMI 1640 medium was purchased from GIBCO (Grand Island, NY). Lymphoprep was purchased from Nycomed AS (Oslo, Norway).

Plasmid construction. pKGE346 (Fig. 1*A*) is a 5-kb pBR322 derivative carrying the chloramphenicol acetyl transferase gene expressed from the hCMV promoter and terminated by the SV-40 polyadenylation signal (provided by Peter Lind, KabiGen, Stockholm, Sweden). To generate CD2 expression plasmids, the CD2 cDNA was obtained as an *Eco*RI fragment and blunt-end ligated into the *Xho*I and *Bal*I sites of pKGE346. The resulting plasmids, phCMV:CD2α (Fig. 1*B*) and phCMV:CD2β are 6-kb plasmids carrying the full length CD2 cDNA (3) in the sense and antisense orientation, respectively, and transcribed through the hCMV promoter (nucleotides −671, +71) (5). In the sense orientation, the cDNA carries two putative polyadenylation sites in its 3′ untranslated region. The cDNA insert is flanked on the 3′ side by a portion of the CAT gene encoding the carboxyl-terminal end of the protein followed by the SV-40 polyadenylation signal. All DNA was purified by CsCl banding and aliquoted before storage at −20°C, at a concentration of 1 μg/μl TE buffer 10 mM Tris, pH7.6 and 1 mM EDTA, pH8.0.

Human primary B cells purification and cultivation. Lymphoprep-separated mononuclear cells were obtained from fresh human blood provided by the local blood bank. After washing with 0.154 M NaCl the lymphocytes were resuspended in 8 ml of culture medium (RPMI 1640 supplemented with 10% FCS) and 500 μl of anti-CD19 immunomagnetic beads were added (4×10^8 beads/ml). The B cells bound to magnetic beads were washed four times with 0.154 M NaCl using a magnetic particle concentrator. B cells and magnetic beads were then resuspended in 10 ml culture medium and incubated overnight at 37°C to allow shedding of the beads. Yields were generally 3 to 5×10^7 B cells per 500-ml blood sample, and contained less than 0.5% $CD2^+$ cells. The cells were cultivated at 1×10^6/ml and, where indicated, supplemented with PDB (5 ng/ml), ionomycin (1.25 μg/ml), BCGF (10%), or IL-2 (20 U/ml).

Electroporation. For each electroporation, 10^7 cells were resuspended in 350 μl of culture medium and transferred to a 1-ml electroporation cuvette with a 0.4-cm space between the electrodes (Bio-Rad, Richmond, CA). After addition of 10 μg plasmid DNA (1 μg/μl stock), the samples were gently shaken, and then kept at room temperature for 5 min. Unless otherwise indicated, the samples were subjected to electroporation at 960 μF and 340 V (850 V/cm) with a Gene Pulser apparatus (Bio-Rad) that generated an exponential decay pulse with a time constant of 23 ± 2 ms. Under these conditions, cell survival, determined 24 h after electroporation using the trypan blue exclusion method, was 14 ± 6%. After electroporation, the samples were kept at room temperature for 5 min before being

[3] Abbreviations used in this paper: PDB, phorbol 12,13-dibutyrate; BCGF, B cell growth factor; CAT, chloramphenicol acetyl transferase; hCMV, human cytomegalovirus.

diluted with 10 ml culture medium.

CD2-expression analysis of transfected cells. For analysis of cell surface CD2 expression, cells were stained with fluorescein-conjugated anti-Leu 5b antibodies according to the instructions of the supplier. The cells were then washed twice with ice-cold PBS and resuspended in 400 μl ice-cold PBS. Propidium iodide was added to a final concentration of 10 μg/ml. The cells were analyzed on a flow cytometer (FACscan, Becton Dickinson) gated on side and forward light scatter as well as for propidium iodide emitted fluorescence so as to monitor only live cells. For each sample at least 3000 live cells were counted.

Purification of CD2 transfectants using magnetic beads or panning. For the isolation of $CD2^+$ cells, PDB/ionomycin pretreated cells were electroporated in the presence of 10 μg each of pKGE346 and phCMV:CD2α. Then 20 h after electroporation, the cells were incubated on a rotating shaker for 30 min at 4°C in 10 ml of culture medium containing 400 μl of anti-CD2 immunomagnetic beads (4×10^8 beads/ml), after which time the supernatant was transfered to a fresh tube. The cells bound to beads were then washed twice by magnetic separation and resuspended in 0.154 M NaCl. An aliquot was taken for cell counting although the remaining sample as well as the supernatant were concentrated once more before resuspension in 100 μl of 0.25 M Tris pH 7.5 in preparation for the CAT assay.

Alternatively, panning (6) was used to purify $CD2^+$ cells. The electroporated cells were concentrated in 3 ml medium and transferred to a 10 ml plastic culture bottle (Nunc, Roskilde, Denmark) precoated with OKT11 antibodies purified from ascites. The bottles were then centrifuged on a microtiter plate holder for 10 min at 200 × *g* and further incubated at room temperature for 50 min. The supernatant was carefully transferred to a fresh tube, and the bottle gently washed twice; the first wash was added to the supernatant. Finally the cells bound to the surface were resuspended in medium using a rubber policeman and an aliquot was taken for counting. The rest of the sample and the supernatant were concentrated and resuspended in 100 μl of 0.25 M Tris pH 7.5 in preparation for the CAT assay.

CAT assay. The method of Gorman et al. (7) was used to determine CAT activity with the modification that, when necessary, the cells collected in 100 μl of 0.25 M Tris, pH 7.5, were sonicated in the presence of magnetic beads. The spots were quantified using a densitometer after 3, 12, and 48 h autoradiography of the separated acetylated chloramphenicol forms using X-OMAT AR films (Eastman Kodak, Rochester, NY).

Measurements of ^{3}H-thymidine incorporation. A total of 96 h after electroporation of cells, three 200-μl aliquots were taken from each 10 ml culture and dispensed into microtiter wells. Cells were incubated for 5 h at 37°C, in the presence of ^{3}H-thymidine (5 μCi/ml), then harvested onto glass filters. Incorporated radioactivity was determined by liquid scintillation.

RESULTS

Effect of voltage. Initial experiments were focused on determining the best voltage of electroporation to obtain transient CD2 expression in PDB and calcium ionophore (ionomycin) activated primary B cells. As shown in Figure 2, at the optimal voltage (340 V) nearly 30% of the phCMV:CD2α transfected B cells expressed CD2 at the cell surface, as determined by flow cytometric analysis.

It is also shown in Figure 2 that the cell viability 24 h after pulse delivery decreased from 20% to less than 0.5% between voltages of 300 and 380 V, respectively. In this specific set of experiments, viability at the optimal 340 V was about 6%, which was lower than our average value (see *Materials and Methods*).

Figure 3*A* shows that the level of expression of surface CD2 among the cells transfected at the optimal voltage varies continuously over three orders of magnitude above background staining, possibly reflecting the amount of DNA taken up by individual cells. In contrast, if cells are electroporated in the presence phCMV:CD2β (antisense orientation), less than 0.5% of the cells express CD2 (Fig. 3*B*). The same result is obtained when electroporation takes place in the absence of added DNA, or in the presence of irrelevant DNA (data not shown), indicating that the electric pulse per se does not induce expression of the endogenous CD2 gene.

Kinetics of transfected CD2 gene expression and effects of activation conditions before electroporation. In subsequent experiments the kinetics and levels of CD2 expression were determined for cells cultured under various conditions during 18 h before electroporation (Fig. 4). Expression of the transfected CD2 gene is a rapid event such that, in the case of PDB/ionomycin preactivated cells, approximately 15% of the viable cells score positive for cell surface CD2 expression within 6 h after pulse delivery. Maximal levels of CD2 expression (30%) are reached by 18 h and maintained until about 48 h post-pulse. Thereafter, the expression slowly declines.

In cell pretreated with PDB alone, expression of CD2 occurs more slowly and at lower levels, being roughly 3% at 6 h post-pulse, and peaking at less than 15%, 18 h post-pulse. Ionomycin or medium pretreated cells were even less competent at expressing the transfected CD2 gene, as demonstrated by their low peak expression of less than 4 and 2%, respectively.

By using flow cytometric analysis, "activated" and "unactivated" B cells can be distinguished due to distinct side and forward light scattering properties (Fig. 5). The flow cytometer allowed us to specifically determine CD2 expression in the small fraction of activated cells (about 10% of viable cells, 6 h post-pulse) present among the PDB, ionomycin, or medium pretreated cells. The data show that these cells have kinetics and peak levels of transient CD2 expression close to that of PDB/ionomycin

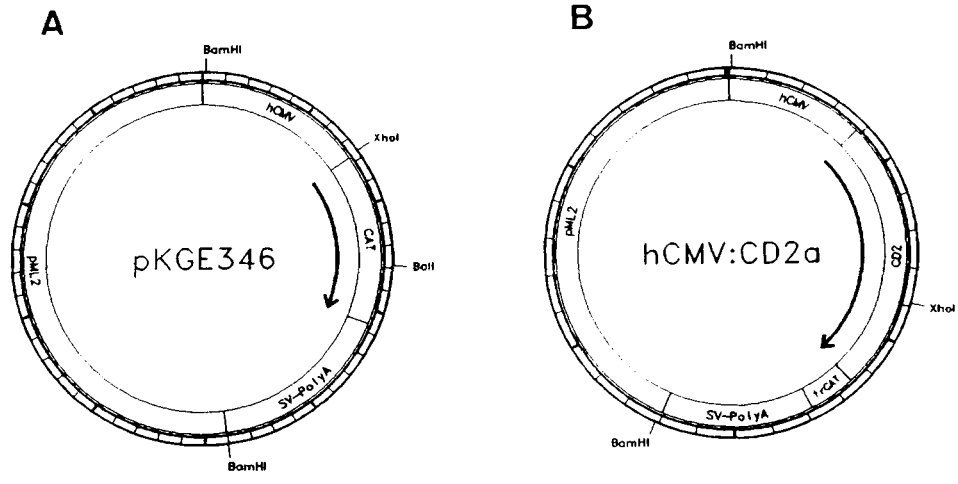

Figure 1. DNA constructs pKGE346 (*A*) and phCMV:CD2α (*B*). pML2 is the pBR322 derived bacterial vector carrying ampicilin resistance and origin of replication; hCMV is the human cytomegalovirus enhancer-promoter element; CAT is the gene encoding the chloramphenicol acetyl transferase; CD2 is the complete CD2 cDNA with its polyadenylation signals; trCAT represents roughly the 260 3′-most nucleotides from CAT; and SV-PolyA is the SV40 polyadenylation signal.

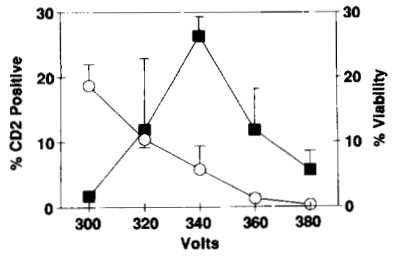

Figure 2. Effect of varying voltage of electroporation on subsequent CD2 expression and cell viability. The cells were cultured in medium supplemented with PDB and ionomycin for 18 h before electroporation in the presence of 10 μg of phCMV:CD2α. The percentage of $CD2^+$ (■) and viable cells (○) were determined 24 h after electroporation. Each *point* is the average of at least three independent electroporations and the *bars* show SD. The time constants for electroporations at 300, 320, 340, 360, and 380 V were, respectively, 27.0 ± 1.4, 23.4 ± 1.7, 23.0 ± 2.2, 25.6 ± 1.6, and 26.1 ± 1.5 ms. The large variation in CD2 expression obtained when electroporation was performed at 320 V resulted from variations in the time constant at a near optimal voltage. Thus, at 320 V a time constant of nearly 25 ms resulted in high CD2 expression (about 20%) whereas smaller time constants resulted in lower CD2 expression.

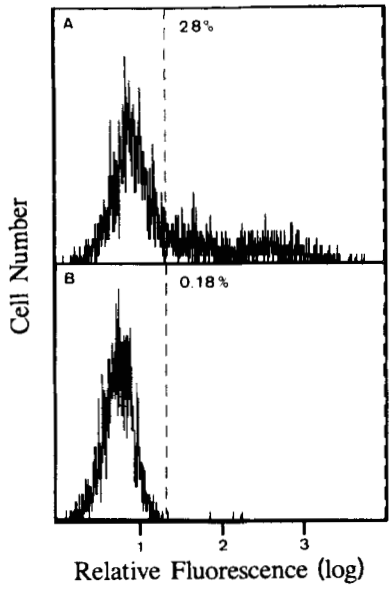

Figure 3. Flow cytometer analysis of CD2 cell surface expression on human primary B cells electroporated in the presence of 10 μg of phCMV:CD2α (*A*) or phCMV:CD2β (*B*). The cells were cultured in medium supplemented with PDB and ionomycin for 18 h before electroporation and cultured in medium containing BCGF and IL-2 after electroporation. The relative number of cells is shown on the *vertical axis* whereas the relative amount of emitted fluorescence for each cell is shown on the *horizontal axis*.

pretreated cells, although with somewhat lower and less durable peak levels of expression (*inset*, Fig. 4; data not shown for PDB-treated cells). These results demonstrate that CD2 is mainly expressed on cells with an "activated" phenotype after electroporation. All cells pretreated with PDB and ionomycin became rapidly activated and no subpopulations of cells expressing higher or lower levels of CD2 Ag could be distinguished on the basis of side and forward light scattering properties.

Effect of activation and culture conditions after electroporation. The results outlined above suggest that transfected CD2 is poorly expressed on unactivated B cells. This apparent lack of cell surface expression of transfected CD2 can be due to low DNA uptake and/or low transcriptional and translational activity in unactivated B cells. To determine whether these cells take up DNA after electroporation, the following experiment was performed: cells cultured in medium alone for 18 h were electroporated in the presence of phCMV:CD2α DNA and subsequently cultured in activation medium supplemented with PDB, ionomycin, and BCGF. As shown in Table I, about 6 to 7% of these cells expressed CD2 on their surface, compared to less than 2% for cells treated similarly before electroporation but not activated after pulse delivery. Moreover, electroporated B cells grown in the presence of PDB and ionomycin were found to become fully activated and proliferating, as determined by ^{3}H-thymidine incorporation measurements (Table I). Thus, the data in Table I show that a significant fraction of resting B cells are also competent in taking up DNA, which may be expressed after cellular activation.

To investigate the influence of growth factors on transient CD2 gene expression in primary B cells, cells treated for 18 h with PDB were electroporated in the presence of hCMV:CD2α and thereafter cultured in medium supplemented with either BCGF, IL-2, BCGF together with IL-2, or in medium alone. Transfected cells were analyzed by flow cytometry after 24 and 48 h of culture. As shown in Table I, neither BCGF nor IL-2 had a direct effect on the transfection efficiencies. However, BCGF appeared to be important for survival of the cells as shown by viability determination 3 days after electroporation (Table I).

Transient cell surface expression of CD2 can be used to enrich for cells co-transfected with CAT expression vector. Transient cell surface expression of the CD2 molecule may be used to selectively purify the cells that have taken up and expressed cotransfected genes. To evaluate this possibility we electroporated PDB/ionomycin-treated cells in the presence of 10 μg of phCMV:CD2α DNA and 10 μg of pKGE346. Then 24 h after electroporation, magnetic beads or plastic culture bottles coated with anti-CD2 antibodies were used to purify cells expressing CD2 on their surface. Both methods allowed recovery of 20 to 30% of the live cells, which is consistent with the observed percentage of $CD2^+$ cells. CAT assays were performed with the lysates from either purified $CD2^+$ cells, nonadherent $CD2^-$ cells, or unpurified transfected cells (Fig. 6). The results revealed that both purification approaches resulted in a large enrichment for CAT activity per μg of protein. When using magnetic beads, the enrichment for CAT activity in the absorbed versus the nonabsorbed cells was 20-fold or more, as determined by densitometer analysis of the autoradiograms.

DISCUSSION

We have described an electroporation protocol for human primary B cells and the use of the CD2 encoding gene as a sortable reporter gene. Under our optimized conditions the transfection efficiency achieved with the activated human primary B cells was comparable to that reported for human lymphoblastoid cell lines although viability might be lower (8). It also appears that the transfection efficiency obtained in the present study was higher than the efficiency achieved using other transfection methods with mouse primary lymphocytes (9) or with human primary B cells (10). However, a quantitative comparison is not possible because CAT assays and S1 nuclease protection assays, respectively, were used in these reports to monitor transient expression.

A significant finding in the present study is that resting unactivated B cells are also competent in taking up DNA. Our criteria for DNA uptake by resting B cells was CD2 expression after PDB and ionomycin activation of elec-

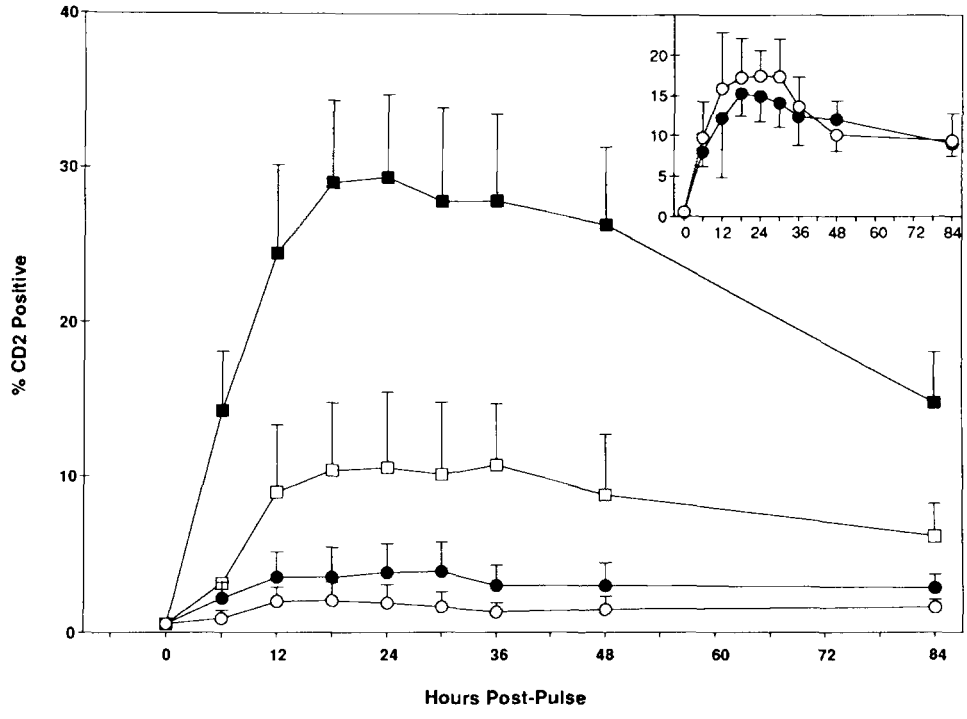

Figure 4. Kinetics of cell surface expression of the CD2 gene and effect of culture conditions before electroporation in the presence of phCMV:CD2α. The cells were cultured in medium alone (○) or medium supplemented with PDB and ionomycin (■), PDB alone (□), or ionomycin alone (●) for 18 h before electroporation in the presence of 10 μg of phCMV:CD2α, and cultured in medium containing BCGF and IL-2 after electroporation. At each time point after electroporation, a 1-ml aliquot was taken from each sample for analysis by flow cytometry as shown in Figure 1. In the case of treatments with PDB, ionomycin, or medium alone, only the major population displayed on a forward scatter vs side scatter dot plot was analyzed for CD2 expression. Each point is the average of at least three independent electroporations; the bars show SD. The insert shows CD2 expression analysis of the small activated subpopulations among ionomycin and medium alone treated cells.

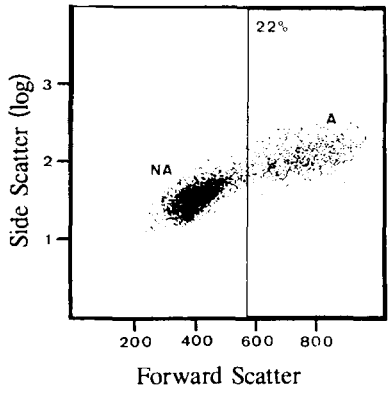

Figure 5. Flow cytometric analysis of 3000 cells showing two distinct populations of human primary B cells 84 h after electroporation. The cells were grown in medium alone for 18 h before electroporation and grown in medium supplemented with BCGF and IL-2 after electroporation. Light side scattering is shown on the *vertical axis* and light forward scattering is shown on the *horizontal axis*. Nonactivated (*NA*) and activated (*A*) cell populations are indicated.

troporated resting cells. It has been proposed that cells in metaphase may be more competent in DNA uptake by electroporation because in such cells the nuclear membrane may be either absent or more permeable. Potter et al. (11) have previously shown that an 18-h colcemide treatment of mouse pre-B lymphocytes before electroporation may increase the number of stable clones by one order of magnitude. Although our results deal with transient expression, it is interesting in this context that the resting unactivated B cells, as well as the PDB-activated but nonproliferating B cells, were clearly shown to be competent in DNA uptake after electroporation (Table I). This may be important for studies aimed at identifying promoters that are active in resting or nonproliferating B cells.

The data presented in Figure 2, *inset*, show that the few activated cells present among cells maintained in medium alone, or medium supplemented with ionomycin, are almost as competent to express the transfected CD2 gene as the PDB and ionomycin-activated and vigorously proliferating cell population. This suggests that the role of these drugs in terms of transient expression of the CD2 gene, which was under the control of the hCMV promoter, is most likely to be restricted to cellular activation. Thus, expression of the transfected DNA seems to be dependant on cellular activation whereas it is ap-

TABLE I

Effects of growth conditions after electroporation on CD2 expression, cell viability, and proliferation[a]

DNA	Factor Before	Factor After	Percent $CD2^+$ 24 H	Percent $CD2^+$ 48 H	No. Cells 96 H	cpm/Cell 96 H
phCMV:CD2α	None	BCGF, PDB, Ionomycin	5.7 ± 1.4	7.2 ± 2.1	478 ± 189	1747 ± 292
phCMV:CD2α	None	None	1.5 ± 0.4	1.4 ± 0.5	125 ± 31	8 ± 5
phCMV:CD2β	PDB	BCGF, IL-2	0.2 ± 0.1	0.4 ± 0.3	194 ± 120	36 ± 6
phCMV:CD2α	PDB	BCGF, IL-2	9.4 ± 0.5	9.4 ± 0.2	171 ± 108	36 ± 5
phCMV:CD2α	PDB	BCGF	9.8 ± 0.8	9.4 ± 0.3	162 ± 73	46 ± 29
phCMV:CD2α	PDB	IL-2	9.0 ± 1.7	9.2 ± 1.0	52 ± 19	6 ± 9
phCMV:CD2α	PDB	None	9.4 ± 3.6	9.9 ± 2.9	37 ± 10	25 ± 17

[a] The cells were cultured for 18 h in medium containing the indicated factors before electroporation in the presence of 10 μg of phCMV:CD2β or phCMV:CD2α, and then cultured in medium supplemented with the indicated factors after electroporation. A 1-ml aliquot was taken from each sample for cell surface CD2 expression analysis at 24 and 48 h after electroporation. In each case only the major population displayed on a forward scatter vs side scatter dot plot was analyzed and the percentage of $CD2^+$ cells determined. Then 96 h after electroporation, viability was determined and is expressed as the total number of surviving cells ($\times 10^{-3}$). Thymidine uptake was also measured 96 h after electroporation during a 5 hours pulse. Triplicate 200-μl aliquots from each electroporation were used and the results are expressed as counts per minutes per cell ($\times 10^3$). At least three independent electroporations were performed in each case.

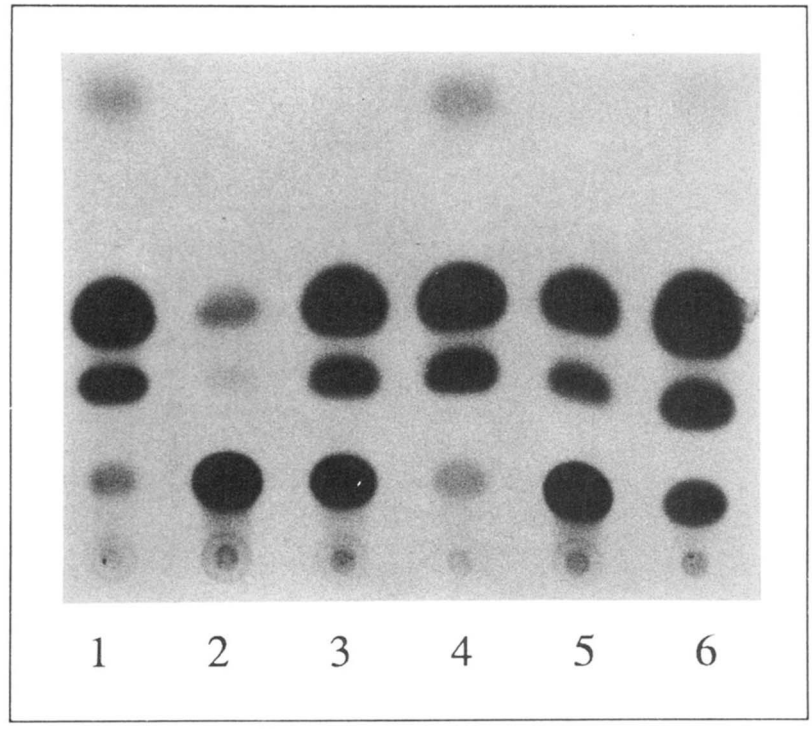

Figure 6. CAT activity in CD2 separated CD2/CAT co-transfected cells. PDB and ionomycin activated cells were electroporated in the presence of 10 μg each of phCMV:CD2α and pKGE346 and subsequently separated on the basis of cell surface CD2 expression. Cell lysates were then assayed for CAT activity. *1*, Cells purified using anti-CD2 coated magnetic beads; *2*, supernatant from the immunomagnetic separation; *3*, total cells used in the separation shown in *lanes 1* and *2*; *4*, cells purified by panning; *5*, supernatant from panning separation; 6, total cells used in the separation shown in *lanes 4* and *5*.

parently less important that the B cells be actively proliferating.

The relatively rapid cell-surface expression of the transfected CD2 gene combined with the possibility of purifying the transfected cells should allow studies on the effects of genes of interest in transient assays in human primary B cells. In this respect, the CD2 gene itself is interesting. Triggering of CD2 on T cells by appropriate antibodies elicits rapid biochemical changes including breakdown of inositol phospholipids, elevation of cytoplasmic free calcium, and activation of protein kinase C. However, signal transduction via CD2 appears to be dependent on the interaction of CD2 with other molecules present on the T cell membrane, such as the CD3-TCR (12, 13). CD2 expressed on the surface of insect cells, or on murine L cells, has been shown to be devoid of signal transducing properties (14, 15). Inasmuch as B and T cells are closely related cell types, it would be interesting to determine whether CD2 is functional or inert on the surface of human primary B cells, especially as CD2 has recently been shown to be expressed on murine B cells (16).

It is evident from the present study that a cDNA encoding a cell surface molecule might be a convenient type of reporter gene in transfection studies since it allows the use of monoclonal antibodies, combined with flow cytometry, for detection of gene expression at the single cell level. Inasmuch as cell surface expression of a reporter gene also allows purification of transfected cells, as shown in Figure 4, it will be possible to study the effects of cotransfected genes in a B cell population that may consist entirely of transfected cells.

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