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Cord blood CD4$^+$ CD45RA$^+$ T cells achieve a lower magnitude of activation when compared with their adult counterparts

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SUMMARY

Highly purified CD4$^+$ CD45RA$^+$ cells from cord blood and peripheral blood from healthy adults were studied. The levels of expression of the CD2, CD3, CD4 and CD28 antigens were similar; however, CD45 and CD45RA antigen expression were slightly lower in cord cells. The reduced expression of the CD45RA antigen on cord CD4$^+$ T cells was confirmed in whole blood. Functional assessment revealed deficiencies in cord CD4$^+$ CD45RA$^+$ T cells. Interleukin-2 (IL-2) production in response to specific triggering via CD2 monoclonal antibody (mAb) alone, or CD2 mAb in combination with CD28 mAb showed marked underproduction (about 10% of adult production). When CD25 expression was examined, it was observed that the proportion of activated CD4$^+$ CD45RA$^+$ T cells in cord blood was lower than in adult (about 20% of adult expression). Proliferation to CD2 mAbs or CD2 + 28 mAbs of cord blood naive cells was similarly depressed. Investigation of IL-2 mRNA expression under these stimulatory conditions paralleled the results observed for CD25 expression, IL-2 production and proliferation. When phorbol 12-myristate 13-acetate (PMA) was added to the cells triggered with CD2 + 28mAbs, the responses examined were enhanced in both cord and adult blood with no significant differences between the groups. These findings suggest that under identical conditions of stimulation, purified cord blood CD4$^+$ CD45RA$^+$ T cells do not acquire similar activation status as their adult counterparts. These finding may help in understanding the reduced graft-versus-host disease (GVHD) observed in cord blood stem cell transplantation.

INTRODUCTION

The majority of CD4$^+$ T cells in cord blood express the CD45RA antigen representative of 'unprimed naive' cells.[1,2] It has been shown that the CD45RA$^+$ T cell population is the subset responding to primary antigenic challenge[3] and to be the major producers of interleukin-2 (IL-2).[4] Previous studies in the literature have assumed that CD4$^+$ CD45RA$^+$ T cells from cord and adult blood are similar with regard to phenotype and function. However, recent reports have shown that adult RA$^+$ cells may comprise reverted RO$^+$[5,6] cells and would suggest that RA$^+$ cells in different microenvironments may differ. Furthermore, neonatal T cells have been shown to express an immature phenotype, CD38 which is present on thymocytes but rarely on adult T cells.[7,8]

In a recent report, we showed reduced precursor frequencies of primary antigen-specific T cells in cord blood using limiting dilution analysis.[9] The aims of the present study were to compare the activation response of highly purified CD4$^+$ CD45RA$^+$ T cells isolated from cord and adult blood employing CD2 and CD28 monoclonal antibodies and phorbol ester. We found that although phenotypically similar, under identical conditions of stimulation, cord CD4$^+$ CD45RA$^+$ cells could not achieve a similar magnitude of activation to their adult counterparts.

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MATERIALS AND METHODS

Reagents

Anti-CD2 antibodies AICD2M1 and AICD2M2 were a gift from Dr Stefan Meuer (Deutsches Krebforschungszentrum, Heidelberg, Germany), and are known to be mitogenic.[10] Anti-CD28 monoclonal antibody (mAb) (9.3) was a gift from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). Phorbol 12 myristate 13-acetate (PMA) was purchased from Sigma, (Poole, UK). All other antibodies were purchased from Dakopatts (High Wycombe, Bucks, UK) or Becton Dickinson (Mountain View, CA).

Cell purification

Cord blood was collected from the umbilical vein, immediately after delivery, in uncomplicated pregnancies at term (mean gestation = 40 weeks, range 38–41 weeks). Venous peripheral blood was obtained from healthy adult volunteers. Mononuclear cells were isolated by Ficoll–Hypaque (Lymphoprep, Nycomed, Norway) density gradient centrifugation and resuspended in complete culture medium.

Peripheral blood mononuclear cells (PBMC) were depleted of adherent cells by incubation in plastic tissue culture flasks (Nunclon, Denmark) for 60 min at 37°. $CD4^+$ $CD45RA^+$ T cells were prepared by negative selection from non-adherent cells. Three rounds of centrifugation using antibody coated magnetic beads (Dynal A.S., Oslo, Norway) were performed to deplete the $CD8^+$, $CD45RO^+$, $CD19^+$, $CD14^+$ and $HLA\text{-}DR^+$ cells. The resulting populations were >99% viable, >98% $CD3^+$, >97% $CD4^+$, and >98% $CD45RA^+$; they contained <1·0% $CD8^+$, $CD16^+$, $CD45RO^+$, $CD19^+$, $CD14^+$ and $HLA\text{-}DR^+$ cells. Purity was confirmed by non-responsiveness to phytohaemagglutinin (PHA) at a final concentration of 2·5 μg/ml.

Cell culture

All cell cultures were carried out in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with L-glutamine (2 mM), gentamycin (50 μg/ml), fungizone (2 μg/ml), and 10% heat-inactivated fetal calf serum (FCS). $CD4^+$ $CD45RA^+$ T cells (2×10^5/well) were cultured in triplicate in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark). Cell proliferation was obtained by adding the combination of anti-CD2 antibodies at 0·25 μg/ml final concentration. Anti-CD28 mAb was used at 1:1000 dilution. PMA was used at 50 ng/ml. The cultures were incubated in a 5% CO_2 humidified atmosphere at 37° for 72 hr. Cell proliferation was measured by ^{3}H-thymidine incorporation (0·3 μCi: specific activity 25 Ci/mmol: Amersham, UK) during the last 18 hr of culture and counted in a liquid scintillation counter. Results are expressed as the mean count/min ^{3}H-thymidine incorporation.

Determination of IL-2 synthesis

Naive T cell cultures were set up in 1 ml volumes. After 18 hr of culture, cells were pelleted. Supernatants were stored at −20° until IL-2 levels were measured using an enzyme-linked immunosorbent assay (ELISA; Duoset, Genzyme, Kent, UK).

RNA extraction and slot blot analysis

$CD4^+$ $CD45RA^+$ cells were stimulated for 18 hr. Cytoplasmic lysates were prepared for mRNA analysis as previously described.[11] Adult and cord $CD4^+$ $CD45RA^+$ T cells were stimulated and processed in parallel in each experiment.

Immunofluorescence studies

Surface expression of CD25 antigen levels was assessed by direct immunofluorescence staining of purified $CD4^+$ naive T cells which had been cultured overnight at 37° either alone, stimulated using anti-CD2 mAbs, anti-CD2 + CD28 or anti-CD2 + CD28 + PMA.

Statistical analysis

Statistical analysis was performed using the Wilxocon rank test for non-parametric data.

RESULTS

Flow cytometric analysis of purified $CD4^+$ $CD45RA^+$ T cells from cord and adult blood

Two types of naive T cells were used throughout this study:[1] purified cord blood $CD4^+$ $CD45RA^+$ T cells, and[2] purified adult $CD4^+$ $CD45RA^+$ T cells isolated from umbilical cord blood and adult peripheral blood, respectively. We first examined the expression of the cell surface antigens CD2, CD3, CD4, CD45RA, CD45 and CD28 on cord and adult naive T-helper cells using two-colour flow cytometry. Table 1 shows that no differences were observed when the mean fluorescence intensity (MFI) or median channel number were compared for the CD2, CD3, CD4 and CD28 antigens. In contrast, CD45 and CD45RA antigens had lower MFI and median expression on cord cells. These results suggest that overall on a cell per cell basis, cord cells have lower expression of the CD45 antigen. Parameters such as size (forward scatter) and granularity (size scatter) as assessed by flow cytometry were similar in both adult and cord cells (data not shown). Unseparated whole blood analysis of the $CD4^+$ $CD45RA^+$ T cell sub-population in 10 adults and 10 cord bloods confirmed our results. Examination of the CD45RA antigen on $CD4^+$ T cells in whole blood analyses showed that cord cells had significantly lower expression as assessed by MFI ($P<0.001$), median ($P=0.001$) and peak channel number ($P=0.002$).

Table 1. Expression of T-cell antigens on cord and adult $CD4^+$ $CD45RA^+$ T cells

	CD2	CD3	CD4	CD28	CD45	CD45RA
Mean fluorescence intensity (MFI)						
Adult	148·69	103·79	76·21	40·64	278·18	97·27
	±10·49	8·15	6·30	1·93	16·44	7·38
CB	153·02	109·25	70·19	42·32	228·22	80·67
	±5·05	9·18	5·39	5·24	20·28	9·43
Median fluorescence channel number						
Adult	146·11	92·99	75·29	27·01	278·21	84·17
	±7·88	3·58	6·58	2·43	14·96	8·34
CB	156·14	100·21	65·20	26·21	227·62	59·59
	±6·64	8·91	6·21	1·69	18·93	9·24

Data acquired ungated are expressed as mean ±SEM of 20 separate adult and cord bloods. Purified naive T-cell preparations were stained with FITC- or PE-conjugated monoclonal antibodies and 5000 cells were analysed by flow cytometry. Non-parametric statistical analysis of adult versus cord CD45RA and CD45 median channel numbers were $P=0.041$ and $P=0.055$, respectively. All other comparisons of adult versus cord antigen expression were not statistically different.

Induction of CD25 expression on cord $CD4^+$ $CD45RA^+$ T cells is lower than phenotypically identical cells from adults

As shown in Fig. 1, stimulation of cord and adult naive T cells via triggering with CD2 mAbs or CD2 mAbs in combination with CD28 mAb induced a lower number of cord cells to become activated and acquire CD25 expression. CD2 mAbs signalling induced on average 20% of adult cells to express CD25 whereas only 9% of cord cells did so ($P<0.01$). Similarly, stimulation with CD2 + CD28 mAbs activated a higher proportion of adult cells 51% compared with cord naive cells (16% $P<0.01$). In contrast, cord cells stimulated with CD2 + CD28 mAb as well as the phorbol ester PMA showed similar numbers of activated cells (94%) compared with adults (94%). These results suggest that specific activation of cord cells with CD2 and CD28 mAbs is not sufficient to activate

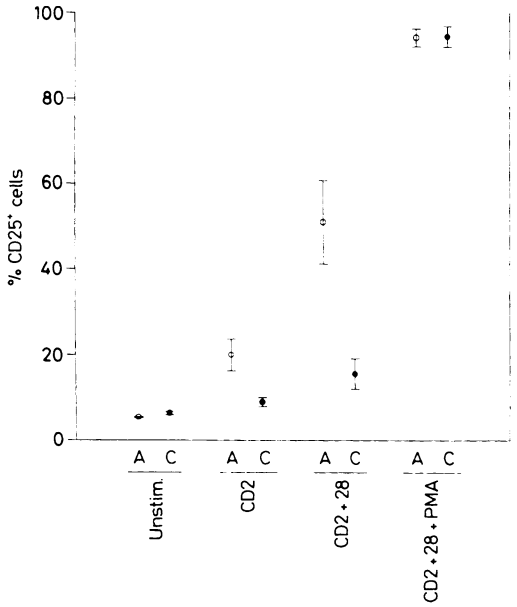

Figure 1. Expression of CD25 by cord and adult naive T cells in the absence (Unstim.) or presence of CD2 mAbs, CD2+CD28 mAbs ±PMA. 5000 cells were analysed for each sample, and were acquired ungated, as in Table 1. These results are the mean of four separate experiments.

all cells whereas direct activation of protein kinase C (PKC) using PMA significantly increases the number of activated cells.

Naive T-cell expression of IL-2 mRNA and IL-2 upon stimulation

Since the major effector function of these cells is restricted to IL-2 production, we examined IL-2 mRNA expression under conditions of stimulation similar to that shown in Fig. 1. Unstimulated cells or CD2 mAbs activated cells showed no detectable IL-2 mRNA expression (Fig. 2) or IL-2 synthesis (Fig. 3). Co-stimulation with CD28 mAb increased IL-2 production in adult cells to 746±205 pg/ml whereas cord cells showed a modest increase to 68±120 pg/ml ($P=0{\cdot}0025$). The accumulation of IL-2 mRNA reflected these results. In the presence of PMA, cord naive T cells showed a greater enhancement in IL-2 mRNA levels and IL-2 production (6601±998 pg ml) when compared to adults (8709± 1060 pg ml). β-actin mRNA expression shown in the lower panel of Fig. 2 was similar for all samples.

Lower proliferative responses in cord naive T cells

In parallel with the IL-2 mRNA levels and IL-2 synthesized, cord T cells again showed reduced proliferative responses to CD2+CD28 mAbs although this did not reach statistical significance ($P>0{\cdot}05$) (Fig. 4). The presence of PMA did not significantly alter the proliferation of cells. Non-responsiveness of $CD4^+$ $CD45RA^+$ T cells to PHA at 2·5 μg/ml final concentration is shown to confirm purity of the cells. A slight response to CD2 mAbs was observed in the adult cells which reflects CD25 antigen expression (Fig. 1).

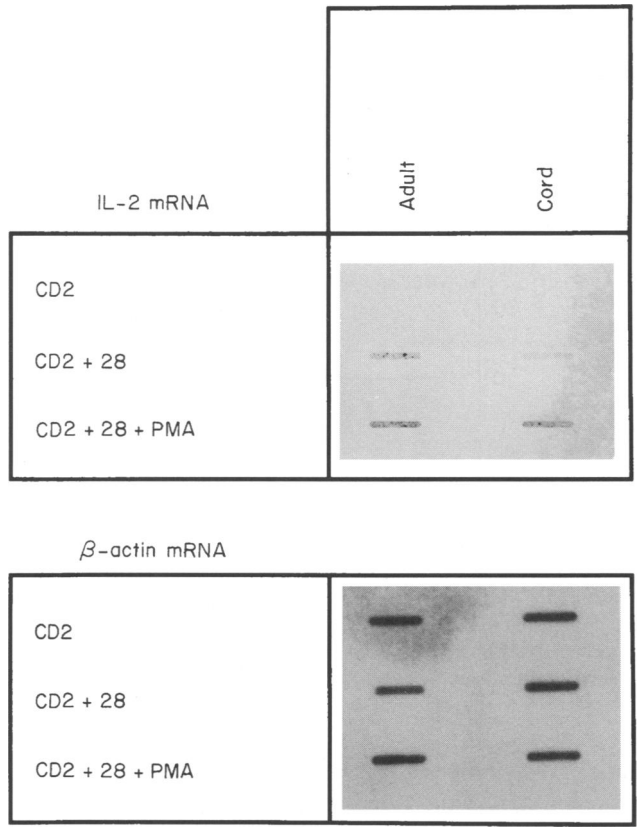

Figure 2. Induction of IL-2 mRNA expression. 5×10^6 purified naive T cells were cultured in the presence of CD2 mAbs, CD2+ CD8 mAbs±PMA. Northern slot blotting was performed on total RNA extracts. IL-2 mRNA is shown in the upper panel and β-actin mRNA in the lower panel and is representative of four separate experiments.

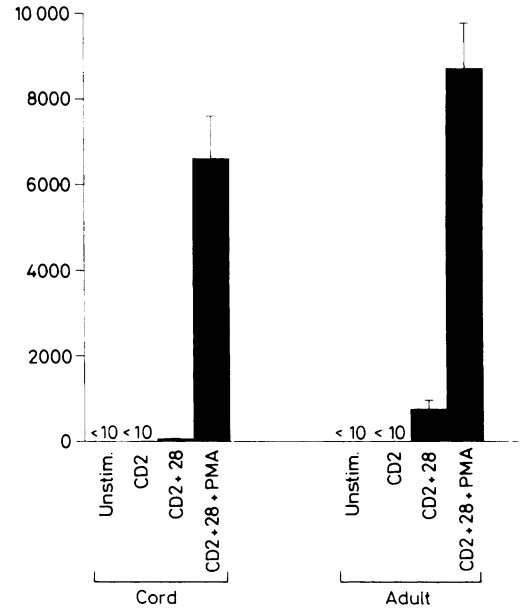

Figure 3. IL-2 production by purified naive T cells in response to CD2 mAbs, CD2+CD28 mAbs±PMA. Supernatants were harvested 18 hr after culture. IL-2 concentrations were determined by ELISA assays. The sensitivity of the assay was 10 pg/ml.

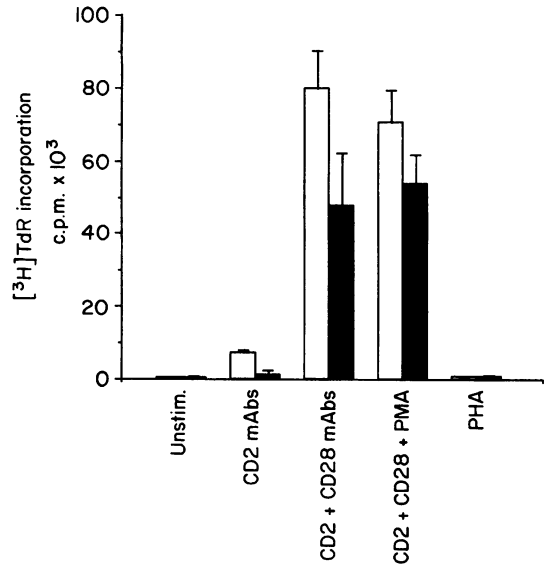

Figure 4. Proliferative responses of purified adult (open bars) and cord (close bars) $CD4^+$ $CD45RA^+$ T cells. Cultures were performed as in the Materials and Methods. Data are representative of the mean ±SEM of four separate experiments.

DISCUSSION

Human cord blood is a rich source of naive $CD45RA^+$ T cells. In the literature, assumptions have been made that because phenotypically similar, adult and cord $CD45RA^+$ T cells are also functionally similar. However, we have recently hypothesized that these cells differ functionally.[12] The present study analyses highly purified $CD4^+$ $CD45RA^+$ T cells from cord and adult blood with regard to activation and the production of IL-2 on the basis of cell equivalence.

We have shown that highly purified naive human $CD4^+$ T cells from cord blood achieve a lower magnitude of activation when compared with phenotypically identical cells isolated from adult peripheral blood. Activated cord $CD45RA^+$ T cells differed from adult T cells in several parameters: the decreased induction of CD25 expression, reduced IL-2 mRNA expression, underproduction of IL-2 and depressed proliferation. Although adult naive $CD4^+$ cells responded vigorously to CD2+CD28 mAbs costimulation, cord T cells responded modestly. However, the addition of phorbol ester enhanced CD25 expression, IL-2 mRNA levels and IL-2 production, although proliferative responses remained unaltered. Overall, PMA appears to up-regulate cord T cell responses to adult-like levels.

The poor responses of cord naive T cell could be accounted for in several ways. Our studies have examined total bulk cultures of highly purified naive T cells. It is possible that a subset of cord T cells is unresponsive and requires additional co-stimulation in order to respond under our conditions of activation. Alternately, adult naive T cells comprise a mixture of 'conventional' RA^+ and reverted RO^+ cells and these latter cells may provide the priming for a response. Another possibility may involve both the active inhibition of IL-2 synthesis and deficiencies in T-cell responsiveness to anti-CD2 stimulation. From previous studies by others[13] and in our laboratory[11] conducted on neonatal T cells, we feel that the underproduction of IL-2 reflects an intrinsic reduced responsiveness of cord $CD45RA^+$ T cells to stimulation via the CD2 molecule. Similar findings have been reported in mice at 24 hr although the early production of high levels of IL-4 were suggested to down-regulate IL-2 synthesis.[14] Human $CD45RA^+$ T cells from adult and cord, however, require repetitive cycles of stimulation for induction of IL-4.[15] However, neonatal $CD45RA^+$ T cells may in the presence of stimuli such as IL-2 and IL-4 be equal to or more responsive than adult RA^+ T cells.[16]

Cord $CD45RA^+$ T cells may not respond if they are in a state of non-responsiveness. This is probably the case since in previous studies addition of exogenous IL-2 to total T cells from cord blood restored the proliferative response to CD2 mAbs to adult T-cell levels.[11] Findings of T cells from neonatal mice of little to no IL-2 synthesis support our studies.[14] Furthermore, the capacity to produce high, adult-like levels of IL-2 is not acquired until later[17] and IL-2 mRNA accumulation in murine T cells has been shown to be developmentally controlled and depends on the maturational state of the T cell.[17] It is possible that maturation of cord blood naive T cells continues in the periphery *in vivo* to progress to mature adult $CD45RA^+$ T cells as have been previously reported in rats.[18]

CD45 isoform expression is highly regulated in lymphocyte differentiation and activation, suggesting that expression of particular isoforms are important to cellular function. The expression of different isoforms in human T lymphocytes has been shown to affect T-cell receptor (TCR) signalling differently.[19–21] However, the possibility that the overall level of CD45 expressed by a cell may affect signal transduction has not been addressed in humans. CD45 dephosphorylates and activates members of the Src family of kinases apparently before cellular stimulation.[22,23] This function is essential for efficient signalling through the antigen receptor of lymphocytes. Activity and expression of $p56^{lck}$ and $p59^{fyn}$ both key members of the Src family have been reported to be deficient in cord $CD4^+$ $CD45RA^+$ T cells compared with the same cell type in adults.[24]

Recent studies have also shown that in anergic murine T-cell clones both the TCR:CD3 complex and the CD4 molecule were expressed at normal levels on the surface, but $p56^{lck}$ was present at a lower level compared to antigen-responsive cells.[25] Furthermore $p56^{lck}$ expression returned to normal after reversal of anergy by growth in IL-2,[25] indicating a correlation between Lck expression and functional anergy. Such studies are currently under investigation in cord naive T cells.

The results in this paper, suggest that the primary T-cell immune response, primarily IL-2, may mature more slowly during development than the adult T-cell response. In support of this idea we have previously observed reduced precursor frequencies to primary antigen in cord T cells and deficient IL-2 production to primary antigen.[9] Recently, considerable interest has arisen as to the use of human cord blood as a source of stem cells for marrow replacement when familial HLA-matched bone marrow donors are not available. To date, over 300 successful cord blood transplants have been performed worldwide. Our studies may partially explain the low incidence and less severe graft-versus-host disease (GVHD) observed. The deficit in IL-2 production observed in cord blood, the lack of help for antibody production[26] and the

defective cytotoxic responses[26] may all aid in the manifestation of transplant tolerance.

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