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Defective Cytokine Expression but Adult-Type T-Cell Receptor, CD8, and $p56^{lck}$ Modulation in CD3- or CD2-Activated T Cells from Neonates

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ABSTRACT

Expression of IL-2, interferon-γ, and IL-3 mRNA and proteins was investigated in peripheral blood mononuclear cells from cord blood after activation with phytohemagglutinin, CD2, or CD3 MAb. The results showed that interferon-γ and IL-3 expression was decreased in cord peripheral blood mononuclear cells when compared with expression observed in adult peripheral blood mononuclear cells, irrespective of the stimulation used. In addition, in newborn cells a defect in IL-2 secretion and mRNA expression was observed in response to CD2 or CD3 MAb but not in response to phytohemagglutinin-mediated activation. We further analyzed the modulation of nonlymphokine genes under the same protocol of stimulations. The results indicate that in newborn cells, despite a reduced lymphokine expression observed after CD2 or CD3 MAb activation, the up-regulation of the T-cell receptor, CD8, and $p56^{lck}$ was similar to that found in adult cells, as was also found after phytohemagglutinin activation of both types of cells. These data are in favor of a deficient T-cell responsiveness to CD2 or CD3 MAb in newborn cells. This impairment of the T-cell response appears to selectively affect lymphokine gene expression because the modulation of other genes also implicated in T cell activation is not altered. (***Pediatr Res* 37: 64–69, 1995**)

Abbreviations

PBMC, peripheral blood mononuclear cell
cPBMC, cord peripheral blood mononuclear cell
PHA, phytohemagglutinin
CD, cluster of differentiation
aPBMC, adult peripheral blood mononuclear cell
TcRα, α-chain of the T-cell receptor
$p56^{lck}$, the $p56^{lck}$ tyrosine protein kinase
IL-2Rα, α-chain of the receptor for IL-2
IFN, interferon

All data reported so far indicate that cytokine production of newborn T cells is different from that observed in adult T cells. Indeed, in cord T cells stimulated with the T-cell mitogen PHA, low levels of IFN-γ, IL-3, and IL-4 have been repeatedly reported (1–6). In addition, IL-2 expression has been shown to be impaired in newborn cells stimulated through the CD2 molecule (7) or through the T-cell receptor complex (8). Nevertheless, the lack of IL-2 in $CD4^+$ cord T cells stimulated with CD3 MAb was not confirmed in a more recent report (1). Therefore, we have decided to compare IL-2, IL-3, and IFN-γ expression in cPBMC and aPBMC in response to PHA and CD2 or CD3 MAb.

We provide definite evidence that newborn cells present, through CD2 or CD3/T cell receptor activation, an altered cytokine expression including that of IL-2. We thus evaluated whether the low responsiveness of newborn cells to CD2 or CD3 MAb also influences the expression of nonlymphokine genes. To address this issue, the expression of the genes encoding the TcRα, the CD8 molecule, and $p56^{lck}$ was compared after activation of adult and newborn cells. These genes were selected because we (9–11) and others (12) had previously shown that expression of these constitutively expressed genes is modulated after CD3 or CD2 MAb stimulation of adult resting T cells or T-cell lines. Our results show that, despite an almost complete absence of cytokine expression in newborn cells in response to CD2 or CD3 MAb stimulation, the kinetics and levels of expression of these genes were similar to those found in adult cells (TcRα, CD8) or even higher in newborn cells ($p56^{lck}$).

These results suggest that newborn cells are characterized by a defect in lymphokine expression including that of IL-2 after stimulation by CD2 or CD3 MAb. This defect appears to be highly selective for cytokine gene expression, because the

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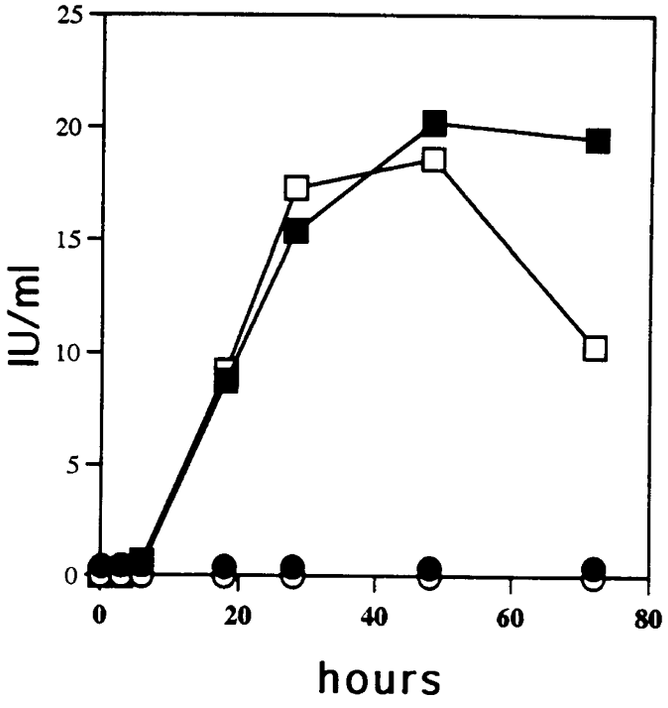

Figure 1. Culture conditions required for precise analysis of IL-2 secretion. PBMC were stimulated with PHA in the presence (■) or absence (□) of a neutralizing MAb recognizing the α-chain of IL-2R added at the beginning of the culture. Culture supernatants were collected at 3, 6, 18, 48, and 72 h after stimulation. Supernatants from unstimulated cells cultured in the presence (●) or absence (○) of the IL-2Rα MAb were collected at the same times and used as controls. Results are expressed as the final concentration of IL-2, measured using a specific immunoassay (ELISA). Results for one of three independently performed representative experiments are shown. Similar findings were observed in the other two experiments.

up-regulation of genes encoding other molecules that also play an important role during T-cell activation is preserved.

METHODS

Reagents. PHA (Wellcome SA, Vallebonne, France) was used at 10 μg/mL. Anti-CD3 MAb (CD3X3) or combinations of two anti-CD2 MAb (CD2X11 and D66 or T11–2 and T11–3) were also used to stimulate T cells. The requirements for T-cell proliferation induced by these MAb in the adult have been extensively characterized elsewhere (13). All antibodies were filtered (0.22 μm) ascitic fluids used at a concentration found to be optimal for stimulation of adult T cells: CD3X3 (1/1000), CD2X11 (1/50), CD2D66 (1/100), T11–2 (1/200), and T11–3 (1/200). In some experiments, when indicated, CD25 MAb (LO-TACT 1 from Dr. H. Bazin, Louvain University, Brussels, Belgium) specific for human IL-2 receptor was also added at a final concentration of 10 μg/mL. This MAb was kindly provided by A. Senick (I.R.S.C, Villejuif, France).

Subjects. cPBMC were collected from healthy full-term neonates immediately after delivery. aPBMC were collected from healthy blood volunteer donors. PBMC were isolated by centrifugation over Ficoll-Hypaque gradients. Cells were washed three times in normal saline and then frozen in nitrogen according to standard procedures until use.

Cell stimulation. PBMC (5×10^5/mL) were cultured in flat-bottomed 24-well plates (1×10^6 cells/well) in RPMI 1640

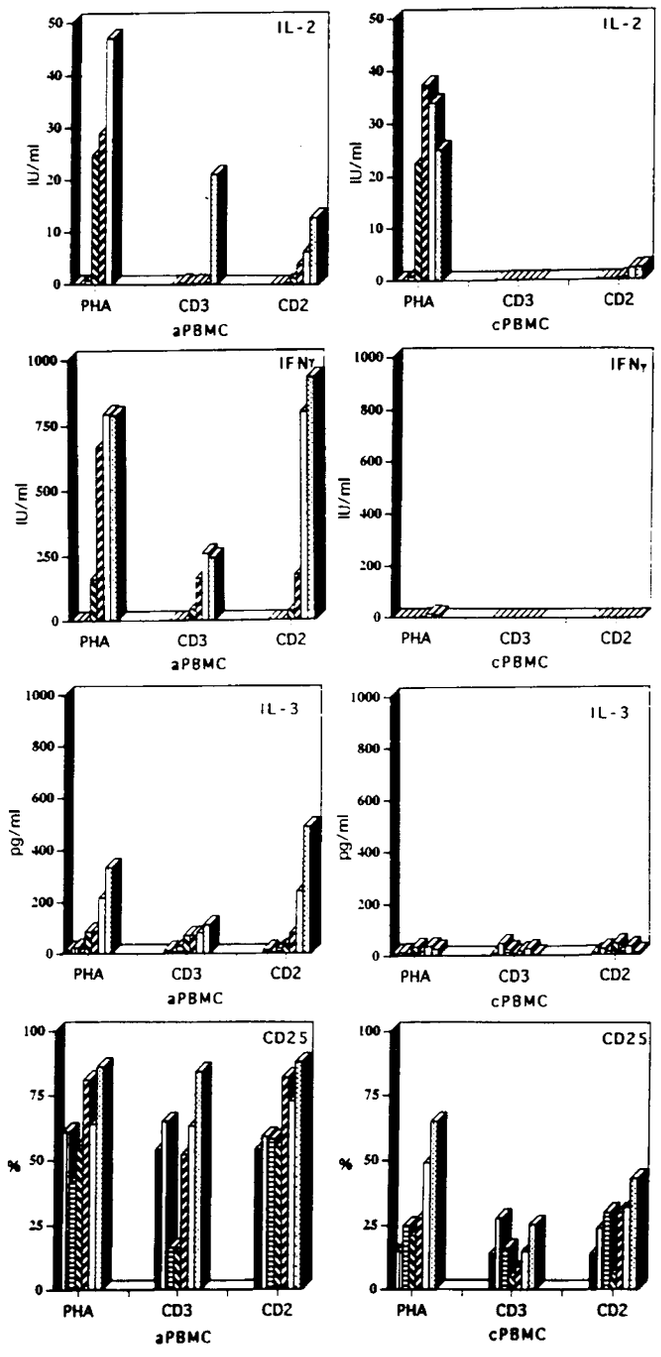

Figure 2. Time course analysis of IL-2, IL-3, IFN-γ secretion, and CD25 expression. aPBMC or cPBMC were stimulated with PHA, CD3 MAb, or a combination of two CD2 MAb (CD2X11 and CD2D66). Culture supernatants were collected at 3 (▥), 6 (▤), 18 (▧), 28 (▨), 48 (□), or 72 (▩) h after stimulation. Supernatants from unstimulated cells were used as control (■). Results are indicated as final concentration of cytokine, measured using a specific immunoassay (ELISA). IL-2R expression was determined by cell labeling with FITC-conjugated CD25 MAb at the indicated times after stimulation. Results are expressed as the percentage of lymphocytes expressing CD25. Three independent experiments were performed. Results of one representative experiment are shown.

culture medium supplemented with 1% glutamine, antibiotics, and 20% heat-inactivated, pooled human AB sera. For stimulation, PHA, CD3, or CD2 MAb were added at the beginning of the culture. The plates were incubated in humidified atmosphere (95% air/5% CO_2) at 37°C.

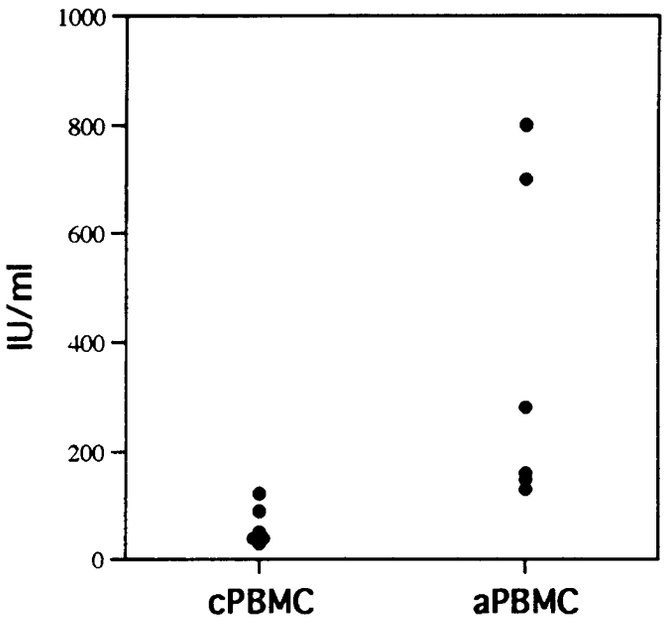

Figure 3. IFN-γ secretion in cPBMC compared with aPBMC. cPBMC (n = 6) or aPBMC (n = 6) were stimulated with PHA. Culture supernatants were collected 48 h after stimulation. Results are indicated as the final concentration of IFN-γ (IU/mL) for each sample, as measured using a specific immunoassay (ELISA).

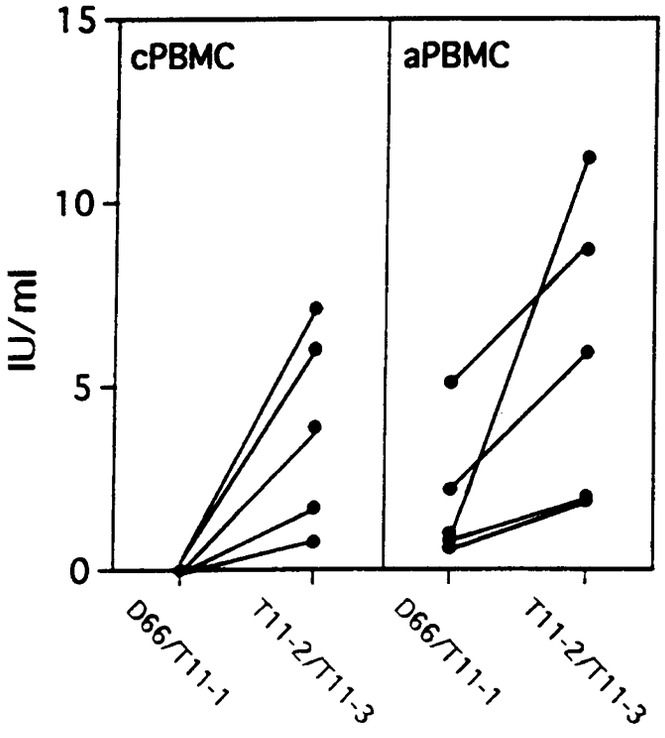

Figure 4. IL-2 secretion after D66/T11–1 and T11–2/T11–3 stimulation. aPBMC (n = 5) or cPBMC (n = 5) were stimulated with D66/T11–1 or T11–2/T11–3 MAb. Culture supernatants were collected 28 h after stimulation. Results for each sample expressed as the final concentration of IL-2, measured with a specific immunoassay (ELISA), are shown.

Analysis of cytokine secretion. Culture supernatants from PBMC activated with PHA, CD3, or CD2 MAb, were collected at 3, 6, 18, 28, 48, or 72 h after stimulation. Supernatants from cells cultured for 1 h in the absence of stimulation were used as a control. Cytokines were measured with specific immunoassays (ELISA) from Medgenix Diagnostics (Fleurus, Belgium) according to the manufacturer's recommendations. Cytokine immunoassay sensitivity was 0.1 IU/mL, 0.03 IU/mL, and 10.5 pg/mL for IL-2, IFN-γ, and IL-3, respectively. Reproducibility was 8, 7.9, and 3.2%, respectively.

Immunofluorescence analysis. PBMC were activated for 3, 6, 18, 28, 48, or 72 h with various agents. After washing, cells were labeled with an FITC-conjugated CD25 MAb (Becton Dickinson, San Jose, CA) as recommended by the manufacturer. Fluorescence of activated cells was analyzed using a FACStar plus (Becton Dickinson) and compared with that of unstimulated cells.

mRNA analysis. Total cellular RNA was extracted by the guanidine isothiocyanate procedure (14) from cells stimulated for 6, 18, and 28 h with the various agents. Total cellular RNA extracted from unstimulated cells was used as a control. Equal amounts of total RNA (10 μg) were fractionated by glyoxal agarose gel electrophoresis and transferred to Gene Screen filters (New England Nuclear, Boston, MA). The filters were sequentially hybridized at high stringency with riboprobes obtained by transcription of T3T7 Bluescribe Vectors (Vector Cloning System, San Diego, CA) containing the following inserts: the 0.3-kb *Xba*I-*Stu*I fragment from human IL-2 cDNA, the 1-kb *Hin*cII fragment from human IFN-γ cDNA (Paul Sondermeyer, Transgene, Strasbourg, France) (15), the 0.38-kb *Pvu*II fragment from the C region of human TcRα, the 1.5-kb *Eco*RI fragment from human CD8 (16), the 0.7-kb *Xho*I-*Hpa*I fragment from human IL-3, the 1.9-kb *Eco*RI fragment from human $p56^{lck}$ (R. Perlmutter, Howard Hughes Medical Institute, Seattle, WA), and the 1.35-kb *Pst*I-*Bam*HI fragment from human IL-2Rα (17). Conditions of transcription were as advised by the manufacturer. Riboprobes were used at 500 000 cpm/mL hybridization medium; after high stringency washes, radioactive signals were detected by autoradiography. Ribosomal RNA was quantified by evaluating the intensity of bands in ethidium bromide–stained agarose gels.

RESULTS

IFN-γ, IL-3, and IL-2 secretion in response to PHA, CD3, or CD2 MAb. The kinetics of IL-2, IFN-γ, and IL-3 secretion was determined in the supernatants of cell cultures at different times after stimulation. In IL-2 secretion assays, an MAb directed against IL-2Rα was added at the onset of the culture to prevent IL-2 consumption and therefore underestimated IL-2 titration (Fig. 1). Figure 2 shows the time course of cytokine secretion in adult and newborn cells. In PBMC of adults, stimulation with PHA, CD3 MAb, or CD2 MAb led to substantial secretion of IL-2, IFN-γ, and IL-3, although IL-2 and IFN-γ secretion was usually greater after stimulation with PHA than after CD2 MAb or CD3 MAb stimulation. In addition, under these three different stimulation conditions, surface expression of IL-2Rα was also observed, as determined by CD25 staining. In PBMC of newborns stimulated with PHA, both IL-2 secretion and IL-2Rα expression were similar to those observed in adults. In contrast, IL-3 and IFN-γ secretion was

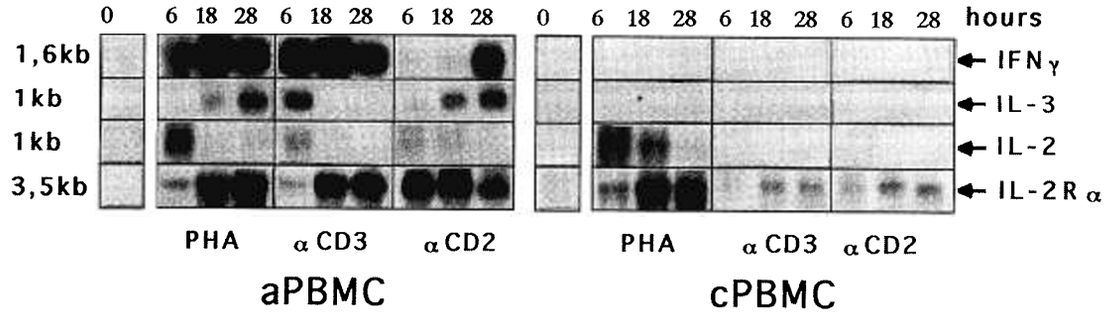

Figure 5. Time course analysis of cytokine and IL-2Rα mRNA expression. aPBMC or cPBMC were stimulated with PHA, CD3 MAb, or CD2MAb. Total cellular RNA was extracted before stimulation (0) or 6, 18, and 28 h after stimulation and analyzed after Northern blot transfer. Filters were sequentially hybridized with the indicated riboprobes specific for IFN-γ, IL-2, IL-3, and IL-2Rα, respectively. The size of the mRNA (in kb) is indicated on the left. Results from one of three independently performed representative experiments are shown.

lower in PHA-stimulated cPBMC compared with that of PHA-stimulated aPBMC. However, it should be noted that IFN-γ secretion after PHA stimulation of cPBMC and aPBMC was quite variable; thus, some overlapping was apparent between the two groups (Fig. 3). Nevertheless, as determined by *t* test, the difference in IFN-γ secretion between cPBMC and aPBMC is significant because the *p* value is less than 0.05. Of note, stimulation of cPBMC by CD2 MAb (D66/T11–1) or CD3 MAb also resulted in a pronounced defect in IL-3 and IFN-γ secretion. In addition, in CD2 or CD3 stimulated cPBMC, a clear defect in IL-2 secretion was observed, and the percentage of cPBMC expressing IL-2Rα was reduced compared with that of aPBMC (Fig. 2).

IL-2 secretion in response to different pairs of CD2 MAb. When T11–2/T11–3 CD2 MAb were used in place of D66/T11–1 CD2 MAb, IL-2 was consistently found in the supernatants of stimulated cord cells (Fig. 4) in agreement with previous reports (7). Differences in IL-2 secretion observed between cPBMC stimulated by D66/T11–1 or T11–2/T11–3 were significant because *p* was less than 0.02. Because T11–2/T11–3 MAb also triggered a higher IL-2 secretion in aPBMC (Fig. 4), the differences observed between these two pairs of MAb in newborns are likely related to the more potent capacity of T11–2/T11–3 to stimulate IL-2 production. Whatever the explanation, these results indicate that the order of defect observed in IL-2 secretion depends on the CD2 pair used to stimulate cord cells.

Cytokine and IL-2Rα mRNA expression. The kinetics of IL-2, IL-3, IFN-γ, and IL-2Rα mRNA expression in response to the various activators was also evaluated. In adults, when PBMC were stimulated with either PHA, CD2 MAb, or CD3 MAb, expression of IFN-γ, IL-2, and IL-3 mRNA was induced (Fig. 5, *left panel*). These stimulatory agents led also to a marked expression of mRNA encoding IL-2Rα. In contrast, almost no cytokine mRNA expression and reduced expression of IL-2Rα mRNA were observed in CD2 MAb- or CD3 MAb-activated newborn PBMC (Fig. 5, *right panel*). However, the accumulation of IL-2 and IL-2Rα mRNA induced by PHA was similar in cPBMC and aPBMC, even though IFN-γ and IL-3 mRNA accumulation was impaired in cPBMC.

TcRα, CD8, and p56^{lck} modulation. We and others (9–12) have previously reported that nonlymphokine genes are modulated in purified adult T cells or in T-cell lines after stimulation by various activators. Whether modulation of these constitutively expressed genes would be observed in newborn PBMC, particularly after CD2 MAb or CD3 MAb stimulation, which resulted in low if any cytokine expression, was to be investigated. To address this point, the membranes used for Northern blot analysis of cytokine expression were sequentially hybridized with TcRα, CD8, and p56^{lck} riboprobes. As shown in Figure 6, TcRα and CD8 mRNA steady state clearly increased up to 18–28 h after activation of aPBMC and cPBMC with PHA, CD2 MAb, or CD3 MAb with similar kinetics. Thus, up-modulation of CD8 and TcRα mRNA was observed in both of these cells after these three activation signals, whether or not lymphokine expression was induced.

The p56^{lck} mRNA accumulation is also shown in Figure 6. Although the constitutive level of p56^{lck} was quite low in both

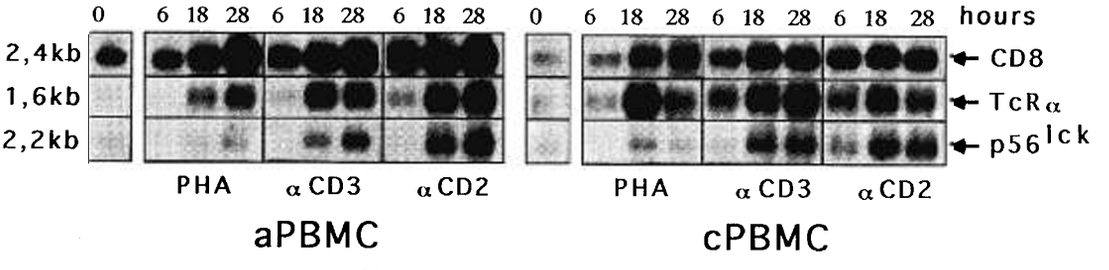

Figure 6. Time course analysis of CD8, TcRα, and p56^{lck} mRNA expression. aPBMC or cPBMC were stimulated with PHA, CD3 MAb, or CD2MAb. Total cellular RNA was extracted before stimulation (0) or 6, 18, and 28 h after stimulation and analyzed after Northern blot transfer. Membranes were sequentially hybridized with the indicated riboprobes specific for CD8, TcRα, and p56^{lck}. The size of the mRNA (in kb) is indicated on the left. Results from one of three independently performed representative experiments are shown.

cPBMC and aPBMC, transient down-regulation of its mRNA observed 6 h postactivation was associated with a strong induction of IL-2 mRNA expression. This early down-modulation was followed at 18 and 28 h by an increased level of $p56^{lck}$ mRNA expression that was always higher than that observed in unstimulated cells. Indeed, this up-regulation of $p56^{lck}$ appeared to be inversely correlated with cytokine mRNA induction, as previously reported (9). In cPBMC, a marked up-regulation of the $p56^{lck}$ mRNA was observed at 18 and 28 h, even in the absence of detectable cytokine mRNA. In addition, in aPBMC and cPBMC stimulated with PHA, the magnitude of $p56^{lck}$ mRNA up-modulation was lower than that observed after CD2 or CD3 MAb activation.

DISCUSSION

In our study, we evaluated the capacity of cPBMC, containing both $CD8^+$ and $CD4^+$ T cells to produce IL-2, IL-3, and IFN-γ in response to various activation stimuli, *i.e.* PHA, CD2 MAb, or CD3 MAb. In agreement with all data reported so far, our results show that PHA-stimulated cPBMC when compared with PHA-stimulated aPBMC expressed decreased amounts of IL-3 and IFN-γ but not of IL-2 or IL-2Rα.

After stimulation by CD2 or CD3 MAb, the defect in IL-3 and IFN-γ expression was more pronounced and was accompanied by both decreased secretion of IL-2 and lower expression of IL-2 Rα. The alteration of IL-2 secretion and IL-2R expression after CD2 MAb or CD3 MAb stimulation clearly resides at a pretranslational level, as shown by the decreased steady state of cytokine and IL-2Rα mRNA accumulation. However, in the case of stimulation via CD2 molecule, the decrease in IL-2 production and IL-2Rα expression depends on the pair of CD2 MAb used. IL-2 was not detected in cells stimulated with the D66/T11–1 pair of CD2 MAb. Nevertheless, in agreement with a previous report (7), we found that the T11–2/T11–3 pair of CD2 MAb led to a substantial level of secreted IL-2. The possibility that T11–2/T11–3 antibodies result in a more potent stimulatory signal than the D66/T11–1 MAb is suggested by two observations. First, in adult cells, IL-2 secretion was always higher after T11–2/T11–3 stimulation when compared with D66/T11–1 activation. Second, we previously reported that accessory signals provided by monocytes are required to induce T-cell proliferation of adult T cells in response to D66/T11–1, whereas they are not for stimulation with T11–2/T11–3 CD2 MAb (13). Together, these results suggest that the partial defect in IL-2 secretion by cPBMC in response to CD2 MAb may be detected only under suboptimal conditions of stimulation. In the case of stimulation via CD3, alterations in IL-2 secretion or mRNA expression by cord cells have been previously reported by Bertotto *et al.* (8) but not by Ehlers and Smith (1). Nevertheless, by comparing the cytokine secretion capability of adult and newborn cells in response to PHA, CD2 MAb, and CD3 MAb, we provide strong evidence for decreased responsiveness of newborn cells to CD2 MAb and CD3 MAb. The mechanisms underlying this defect are still unclear. Inasmuch as cord cells are able to produce IL-2 in response to PHA, the machinery of IL-2 expression is intact in newborn cells. Therefore, absence of IL-2 expression in cPBMC in response to CD2 MAb or CD3 MAb activation is likely to result in altered signal transduction through these molecules.

The major defect in IL-3 and IFN-γ expression and the defect in IL-2 expression in CD2 and CD3 MAb-stimulated cPBMC contrast strikingly with the modulation of TcRα, CD8, and $p56^{lck}$ mRNA levels observed in these cells. Despite the absence of any detectable cytokine mRNA production, the kinetics and levels of TcRα, CD8, and $p56^{lck}$ up-modulation, as measured by Northern blot, were completely normal in cPBMC. These data indicate that defective responses of cPBMC to CD2 MAb or CD3 MAb affect selectively lymphokine gene expression. Furthermore, these results demonstrate that the defect in cytokine production in response to CD2 MAb and CD3 MAb cannot be explained by a general defect of signaling through these two molecules. Thus, these studies support the idea that the signal requirements for mRNA expression and cytokine secretion and those required for the up-modulation of $p56^{lck}$, TcRα, and CD8 are distinct. In the case of IL-2, gene transcription, mRNA translation, and secretion of the protein require the activation of tyrosine kinase, which has been shown to be physically (18–21) and functionally linked to CD3 and CD2 (22, 23). We are currently testing whether the defect in IL-2 secretion by cord T cells stimulated by CD2 MAb or CD3 MAb is associated with particular patterns of tyrosine protein kinase activation or protein phosphorylation.

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