

## Cord Blood T Lymphocytes Lack Constitutive Perforin Expression in Contrast to Adult Peripheral Blood T Lymphocytes

By Christian Berthou, Sabine Legros-Maïda, Annie Soulié, Alain Wargnier, Jacqueline Guillet, Claire Rabian, Eliane Gluckman, and Marilyne Sasportes

**Perforin is the cytolytic pore-forming protein, which alone can be responsible for the lethal hit in one of the killing mechanisms used by natural killer (NK) cells or cytotoxic T lymphocytes. In this study, perforin expression was investigated in cord blood (CB) lymphocytes to determine their killing potential in vivo. The majority of CB CD3<sup>-</sup> NK cells had the protein. Compared with adult perforin-positive NK cells, a significantly lower percentage of cells expressing CD56 and CD57, the related neural cell adhesion molecules, was found ( $P = .0001$ ). Perforin was also present in a unique immature CB NK-cell subset, characterized by cytoplasmic CD3 antigen (Ag) expression. In CB, very few CD8 perforin-positive T lymphocytes could be detected, but they were in**

**significant numbers in adult peripheral blood ( $P = .02$ ). A substantial proportion of these cells ( $70\% \pm 23\%$ ) lacked the CD28 T-cell coactivation Ag, and they were able to exert NK-like, major histocompatibility complex nonrestricted cytolytic activity. CD4<sup>+</sup> and  $\gamma\delta$ -T cells expressing perforin were absent from CB, but low numbers of such cells were detected in adult peripheral blood ( $P = .0001$ ). Therefore, the spontaneous cytolytic activity of CB lymphocytes appeared to be dependent on well-represented perforin-positive NK cells, which were shown to efficiently lyse NK-sensitive target cells.**

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**R**ECENTLY, CRYOPRESERVED umbilical cord blood (CB) cells have been used worldwide in the treatment of 26 children requiring bone marrow (BM) transplantation.<sup>1,2</sup> The results suggest that umbilical CB contains enough hematopoietic stem cells to establish stable engraftment. However, the number of grafted patients at the present time is insufficient to determine if the relative risk of acute graft-versus-host disease after CB transplantation is lower than that observed in patients after BM transplantation.<sup>3</sup> In this context, we investigated the killing potential of CB cells and, especially, the perforin lytic pathway, using antihuman perforin and granzyme B monoclonal antibodies (MoAbs),<sup>4,5</sup> and the cytolytic activity of perforin expressing cells was then examined. Perforin, the pore-forming protein, represents with granzymes, one of the killing mechanisms of cytotoxic T lymphocytes and natural killer (NK) cells.<sup>6,7</sup> According to this model, which is Ca<sup>2+</sup>-dependent, these proteins, which are both found in the granules of cytotoxic effector cells, are released after activation in the intercellular space during effector-target cell interaction.<sup>7</sup> In the current study, we examined 19 umbilical CBs and compared them with the BM from 3 normal donors and the peripheral blood (PB) of 20 healthy adult individuals. Unlike adult PB, CB analysis showed the lack of  $\gamma\delta$ - and  $\alpha\beta$ -T cells of CD8 or CD4 phenotype constitutively expressing perforin. On the

other hand, the presence of a unique immature NK-cell subset absent from adult blood, together with well-represented mature NK cells, both expressing perforin, argued in favor of preferential NK-dependent cell-mediated cytotoxicity in CB.

### MATERIALS AND METHODS

**Cellular fixation and permeabilization.** Mononuclear cells from CB, BM, and PB of healthy adults were isolated by Ficoll-Hypaque density gradient. CBs were obtained after uncomplicated births from Saint-Vincent de Paul and Robert Debre Hospitals (Paris, France); BM cells were obtained from allogeneic transplants (St. Louis Hospital, Paris, France); and human PB were obtained by leukapheresis of normal donors (Blood Bank; St. Louis Hospital). For staining intracellular antigens (Ags), lymphocytes were permeabilized according to the method of Schmid et al.<sup>8</sup> Briefly,  $1 \times 10^6$  washed cells were pelleted and resuspended in 850  $\mu$ L of cold phosphate-buffered saline (PBS). Then, 150  $\mu$ L of cold 4% paraformaldehyde solution was added, and the mixture was immediately vortexed. The samples were incubated at 4°C for 1 hour and centrifuged. The fixed pellet was resuspended in 1 mL of 0.2% Tween 20 and incubated for 15 min at 37°C. The cells were washed with 1 mL of PBS supplemented with 2% fetal calf serum and 0.1% azide. The supernatant was decanted and the samples were processed for intracellular staining.

**Intracellular markers.** Permeabilized lymphocytes were incubated with 50  $\mu$ L of PBS 2% fetal calf serum to reduce nonspecific binding, and 50  $\mu$ L of antihuman perforin or granzyme B MoAbs were added, followed by incubation for 30 minutes at 4°C. After 2 washes in 0.1% Tween in PBS, 50  $\mu$ L of a 1:500 dilution (60 ng) of fluorescein isothiocyanate (FITC)-goat antimouse or 50  $\mu$ L of 1:200 dilution of phycoerythrin (PE)-goat antimouse was added (Caltag Laboratories Inc, San Francisco, CA), followed by incubation for 30 minutes at 4°C. An antitubulin MoAb (personal gift of Dr A. Fellous, INSERM U 96, Bicetre Hospital, Kremlin Bicetre, France) was used as a positive control of cellular permeabilization in all experiments. An FITC- or PE-labeled goat antimouse anti-IgM without the primary antibody or with an irrelevant MoAb was added to CB cells or PBLs and was used as negative control.

**Surface cellular markers.** After two washes in Tween 0.1% in PBS, staining of surface Ags was performed using the following MoAbs: anti-CD3 $\epsilon$  (FITC-, PE-, and PerCP-labeled; Leu4), anti-CD8 $\alpha$  (FITC-, PE-, and PerCP-labeled; Leu2a), anti-CD4 (FITC-, PE-, and PerCP-labeled; Leu3a), anti-CD5 (PE-labeled; Leu1), anti-CD11a (FITC-labeled antihuman  $\alpha$  LFA-1), anti-CD11b (PE-labeled; Leu-15/CR3), anti-CD16 (PE-labeled; Leu1c), anti-CD28

From INSERM U 93, the Laboratory of Immunology and Histocompatibility, Bone Marrow Transplantation Unit, St Louis Hospital, Paris, France.

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Address reprint requests to Marilyne Sasportes, MD, INSERM U 93, Hôpital St Louis, 1 Avenue Claude Vellefaux 75010-Paris, France.

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## LACK OF PERFORIN IN CORD BLOOD T LYMPHOCYTES

(PE-labeled; Leu28), anti-CD56 (PE-labeled; Leu19), anti-CD57 (FITC-labeled; Leu7), anti- $\alpha\beta$ -TcR-1 (FITC-labeled), anti- $\gamma\delta$ -TcR-1 (FITC-labeled), and negative control (Simultest control). All MoAbs were obtained from Becton Dickinson Immunocytometry Systems, Inc (San Jose, CA). After further incubation for 30 minutes at 4°C in the dark and two more washes in PBS, the cell pellets were resuspended in 1% paraformaldehyde.

**Flow cytometry analysis.** Data acquisition and analysis was performed on a Becton Dickinson FACScan flow cytometer equipped with a 15-mW air-cooled 488-nm argon-ion laser. By suitably gating lymphoid populations (forward scatter versus side scatter), dot-plot graphs for dual-Ag expression or three-color fluorescence analysis of  $10^4$  cells were obtained. All immunofluorescence profiles are plotted on a 4-decade log scale. CB lymphocytes (CBLs) were analyzed using two- and three-color fluorescence analysis.

**Purification of NK cells.** CB and adult PB mononuclear cells were prepared by standard Ficoll-Hypaque procedures. After 1 hour of adherence to plastic at 37°C in 5% CO<sub>2</sub>, nonadherent cells were loaded on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 30 minutes at 500g. Cells were recovered from the low-density fraction and purified for NK cells by lysis in presence of anti-CD3 and anti-CD19 hybridomas at 1:100 dilution of an ascite fluid and rabbit complement (Fillorga Laboratories, Paris, France). On purification, the population was analyzed by flow cytometry. NK cells were isolated to greater than 93% purity.

**Cell sorting analysis.** To obtain purified perforin-positive CD8<sup>+</sup> T cells, mononuclear cells were stained on ice with optimal saturating amounts of MoAbs PE-anti-CD5 and FITC-anti-CD57. After staining, cells were washed extensively and purified using flow cytometric cell sorting on a FACStar (Becton Dickinson). The CD5<sup>+</sup> CD57<sup>+</sup> T-cell suspension contained 90% of purified perforin-positive CD8<sup>+</sup> T lymphocytes.

**Cell-mediated cytotoxicity assay.** Cytotoxic activity was measured in a standard 4-hour <sup>51</sup>Cr radioisotope release assay. Resting and activated purified NK cells and perforin-positive CD8<sup>+</sup> T lymphocytes were tested against the NK-sensitive K-562 target and the Daudi cell line, in the presence of phytohemagglutinin (PHA-P; final concentration, 1/600; Wellcome, Beckenham, UK) (lectin-dependent cell-mediated cytotoxicity). Cultures were incubated overnight and for 48 hours at 37°C in 5% CO<sub>2</sub> in RPMI 1640 medium (GIBCO, Paisley, Scotland) supplemented with 10% human serum and 200 U/mL recombinant interleukin-2 (rIL-2; Roussel Uclaf, Paris, France). Targets cells were also added to wells containing medium alone and to wells containing 1% Triton X-100 (Sigma, St Louis, MO) to determine the spontaneous and maximal release, respectively. Assays at various effector-to-target cell (E:T) ratios with  $5 \times 10^3$  <sup>51</sup>Cr-labeled target cells/well were performed in triplicate, using 96-well V-bottom microtiter plates. The final culture volume was 200  $\mu$ L in each well of 96-well microtiter plates. After 4 hours of culture, 100  $\mu$ L was removed from each well and counted in a  $\gamma$ -counter for determination of <sup>51</sup>Cr release. The percentage of specific <sup>51</sup>Cr release was calculated as follows: (cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release)  $\times$  100.

**Statistical analysis.** Results obtained from CB were compared with those from 20 adult PBLs. All experimental values were expressed as a mean percentage of the number of samples studied. The Student's *t*-test one-way analysis of variance was used for comparison of means. *P* < .05 was taken as significant.

## RESULTS

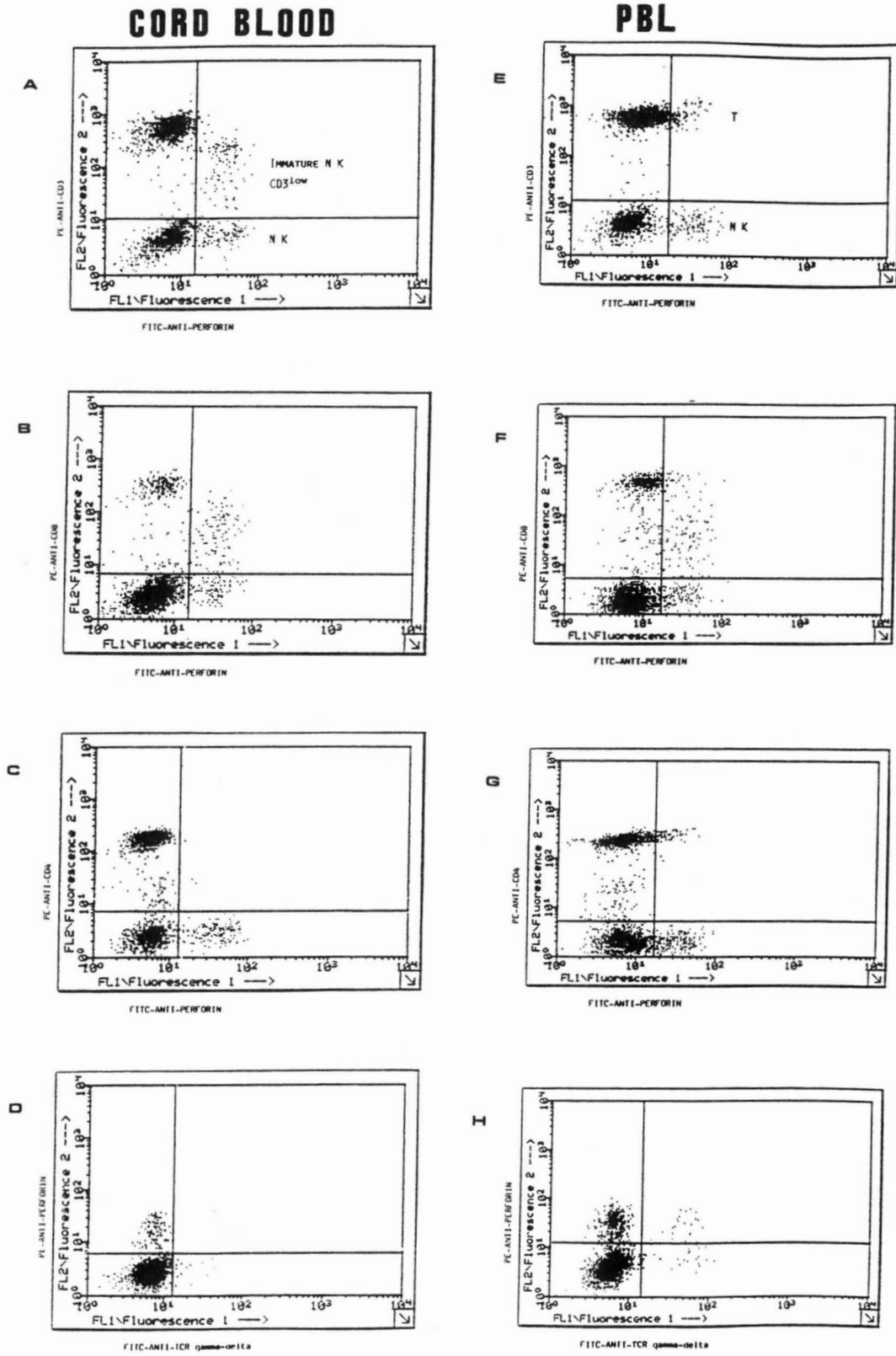
**Perforin expression in CB cell subsets.** In CB, 20%  $\pm$  6% of lymphocytes contained perforin. Based on CD3 ex-

pression, two main populations of perforin-positive cells could be distinguished (Fig 1A). The first subset was composed of CD3<sup>-</sup> NK cells (Fig 1A, lower right quadrant), which represented 14%  $\pm$  7% of total CB lymphocytes (CBLs; see Table 1). Most perforin-positive NK cells expressed CD16, and practically all expressed CD11b (Table 2). CD16 is implicated in the antibody-dependent cellular cytotoxicity (ADCC) function exerted by NK cells, and CD11b is an adhesion molecule from the integrin family. However, only 60%  $\pm$  3% of perforin-positive NK cells expressed the NK marker CD56, and less than 5% were CD57<sup>+</sup> (Table 2). These two Ags are related to neural cell-adhesion molecules. Besides, low levels of CD8 $\alpha$  chains were present on 30%  $\pm$  10% of CB perforin-positive NK cells (Fig 1B). The NK-cell subset with the CD56<sup>+</sup>bright phenotype constantly lacked perforin in all CB tested.

A second subset of CD3<sup>+</sup>low cells was found to express perforin (Fig 1A, upper right quadrant). Analysis of T-cell receptor (TcR) and CD5 expression on these cells was performed using three-color immunofluorescence staining, first by anti-TcR ( $\alpha\beta$  and  $\gamma\delta$ ), anti-CD3, antiperforin, and then by anti-CD5, anti-CD3, and antiperforin MoAbs. CD5 is a T-cell accessory activation Ag, closely associated with the CD3-TcR complex on the surface of human T lymphocytes.<sup>9</sup> The subsequent result highlighted the fact that CD3<sup>+</sup>low perforin-positive cells were TcR- $\alpha\beta$ -, TcR- $\gamma\delta$ -, and CD5-negative, and, in addition, they all lacked CD28 Ag expression. CD28 is a homodimer membrane glycoprotein expressed by peripheral T lymphocytes and thymocytes.<sup>10</sup> Triple-staining analysis of simultaneous expression of CD16, CD3, and perforin was then performed and clearly showed that the majority of CD3<sup>+</sup>low perforin-positive cells bore the CD16 NK-cell associated Ag (Table 2). This subset was further analyzed for cellular localization of CD3 expression. Only CD3 staining of permeabilized lymphocytes allowed CD3<sup>+</sup>low cell subset detection. This proved that these cells expressed cytoplasmic CD3 Ags (cCD3), detected by the MoAb used that recognized the  $\epsilon$  invariant chain of the CD3 complex. We further determined that all cCD3<sup>+</sup>low perforin-positive cells were CD11b<sup>+</sup>; most were CD57<sup>-</sup>; whereas 38%  $\pm$  10% of them had CD8  $\alpha$  chain expression (Table 2). This subset was not detectable in adult PB (see Fig 1A and 1E, upper right quadrant). It represented 5%  $\pm$  2.5% of total CBLs (Table 1).

Analysis of perforin expression in CB CD8<sup>+</sup> T cells was then examined using three-color immunofluorescence cell staining. CD8<sup>+</sup> T cells represented 12%  $\pm$  4% of total CBLs. The vast majority did not express perforin. Some (9%  $\pm$  5%) CD8<sup>+</sup> T cells had the protein; they bore the CD28 Ag and were CD11b- and CD57-negative. CD4<sup>+</sup> T cells represented 38%  $\pm$  7% of total CBLs, whereas few  $\gamma\delta$ -T cells (less than 2%) were detected. Examination of perforin expression in CD4<sup>+</sup> and  $\gamma\delta$ -T cells showed that they lacked perforin (Fig 1C and D).

**Perforin expression in mononuclear adult BM cells.** Mononuclear cells isolated from BM were examined for perforin expression. This protein was detected in CD3<sup>-</sup> NK cells, which represented 3% to 4% of mononuclear cells.



The BM CD3<sup>+</sup> T lymphocytes of CD8 or CD4 phenotype were perforin-negative.

**Perforin expression in adult PB cell subsets.** In adults, 25% ± 12% of PB lymphocytes (PBLs) constitutively expressed perforin, which was not significantly different from CB ( $P = .22$ ). Two populations of CD3<sup>-</sup> and CD3<sup>+</sup> perforin-expressing cells could be detected (Fig 1E). A large fraction of CD3<sup>-</sup> NK cells (73% ± 16%) contained this protein (Table 1). The majority of perforin-positive NK cells expressed low amounts of CD56 (CD56<sup>low</sup>), and 59% ± 14% of them possessed membrane CD57 molecules (Table 2). Higher cell subset expression of these Ags compared with that for CB was very significant ( $P = .0001$ ). CD8  $\alpha$  chain detection on perforin-positive NK cells remained quite similar (36% ± 12%; see Table 2). As in CB, the CD56<sup>bright</sup> NK-cell subset lacked perforin.

Perforin was also detected in CD3<sup>+</sup> T cells. Perforin expression in the CD8<sup>+</sup> T-cell subset was investigated by three-color immunofluorescence analysis composed of anti-CD3, anti-CD8, and antiperforin. A subpopulation of CD8<sup>+</sup> T cells constitutively expressed perforin, which represented 30% ± 17% of the CD8 subset. Both CD28<sup>+</sup> and CD28<sup>-</sup> cells were detected within this population. However, use of three-color immunofluorescence analysis composed of anti-CD28, anti-CD8, and antiperforin allowed us to determine that the CD28<sup>-</sup> subset accounted for 70% ± 23% of the perforin-positive CD8<sup>+</sup> T-cell population (Table 2). These cells were HLA-DR<sup>-</sup> and CD25-negative and, therefore, not activated. They bore CD11b and CD57 Ags and high-density CD11a/LFA1 chains of similar levels to those found on NK cells. Few CD4<sup>+</sup> T lymphocytes also contained perforin (Fig 1G; they represented 6% ± 3% of CD4<sup>+</sup> T cells; see Table 1). Using anti-CD28, anti-CD4, and antiperforin cell staining, 63% ± 20% of perforin-positive CD4<sup>+</sup> T cells were CD28<sup>+</sup>. Perforin expression was also investigated in  $\gamma\delta$ -T cells, where it was detected in 35% ± 18% of them (Fig 1H). A similar proportion of  $\gamma\delta$ -T cells lacked the CD28 Ag.

**Cytotoxic activity of perforin-expressing cells in CB and adult PB.** The cytotoxic activity of purified CB and adult PB NK cells was investigated. As shown in Fig 2, CB NK cells showed a lower level of spontaneous cytotoxicity against K-562, compared with those of adults. However, their level of cytotoxicity increased approximately fourfold after overnight treatment with rIL-2 (76% cytotoxicity) and twofold after 2 days. Furthermore, in the same conditions, they acquired strong cytolytic activity against the Daudi cell line (85% cytotoxicity after overnight culture). After activation, CBNK cells were able to exert similar levels of cytotoxicity compared with those of adults (Fig 2).

**Table 1. Perforin-Expressing Cells in CB and Adult PB**

Cell Subset	CB*	Adult Blood†	P Value
CD3 <sup>-</sup> NK cells			
%/TL	18 ± 6	21 ± 9	.57
% of Perf <sup>+</sup> cells			
/CS	78 ± 11	73 ± 16	.47
/TL	14 ± 7	14 ± 7	.32
CD3 <sup>low</sup> NK cells‡			
%/TL	8 ± 2.5†		
% of Perf <sup>+</sup> cells		ND	
/CS	66 ± 10		
/TL	5.5 ± 2.5		
CD3 <sup>+</sup> $\alpha\beta$ CD8 <sup>+</sup> T cells			
%/TL	12 ± 4	22 ± 10	.01
% of Perf <sup>+</sup> cells			
/CS	9 ± 5	30 ± 17	.003
/TL	1 ± 0.5	7.5 ± 6	.02
CD3 <sup>+</sup> $\alpha\beta$ CD4 <sup>+</sup> T cells			
%/TL	38 ± 7	37 ± 9	.39
% of Perf <sup>+</sup> cells			
/CS	0	6 ± 3	.0001
/TL	0	2 ± 0.9	.0001
CD3 <sup>+</sup> $\gamma\delta$ T cells			
%/TL	1.1 ± 0.5	4 ± 1.7	.0001
% of Perf <sup>+</sup> cells			
/CS	0	35 ± 18	.0001
/TL	0	1.5 ± 0.8	.0001

Abbreviations: TL, total lymphocytes; CS, cell subset; ND, not detected; Perf<sup>+</sup>, perforin-expressing.

\* The data represent mean ± SD of 19 experiments.

† The data represent mean ± SD of 20 experiments.

‡ The percentage of CD3<sup>low</sup> cells was calculated in permeabilized CBL.

Cytotoxicity activity of purified CD8<sup>+</sup> perforin-positive T lymphocytes was then measured. As already mentioned, such cells could hardly be detected in CB. Adult purified perforin-positive CD8<sup>+</sup> T cells (CD57<sup>+</sup>, CD28<sup>-</sup>) had no cytolytic function against K-562 and the Daudi cell line. Examination of these cells after rIL-2 activation showed an efficient cytotoxic activity against K-562 targets and a weak but significant lymphokine-activated killer activity (Fig 2).

**Granzyme B expression in CBLs and adult blood lymphocytes.** Granzyme B expression was detected neither in unstimulated NK cells nor in CD3<sup>+</sup> T lymphocytes from cord and adult bloods.

## DISCUSSION

Lymphocytes expressing perforin function as cytotoxic cells. Inhibition of lymphocyte-mediated cytotoxicity by per-

Fig 1. Comparative analysis of perforin expression in CBL and adult PBL subsets. A representative experiment of 1 CB and 1 adult PB is shown. Cells from randomly selected CB and adult PB were permeabilized as described in Materials and Methods, stained with antiperforin MoAb detected with PE- or FITC-labeled goat antimouse antibodies, as appropriate. Samples subsequently stained with PE-conjugated anti-CD3, anti-CD8, anti-CD4, and FITC-conjugated anti-TcR- $\gamma\delta$  were analyzed by flow cytometry. Data are displayed as dot-plots, and quadrant markers were positioned to include greater than 98% of control Ig-stained cells in the lower left. Correlated expression of perforin versus Ag subset marker is shown. Cells that are double-stained with corresponding PE- and FITC-conjugated antibodies are represented in the upper right quadrant.

**Table 2. Phenotype of Perforin-Expressing Cells in CB and Adult PB**

Cell Subset (CD Ags)	CB (%)*	Adult Blood (%)†	P Value
<b>CD3<sup>-</sup> Perforin<sup>+</sup> NK cells</b>			
CD16 <sup>+</sup>	86 ± 2	92 ± 6	.20
CD11b <sup>+</sup>	97 ± 1	97 ± 3	.17
CD56 <sup>+</sup>	60 ± 3	92 ± 5	.0001
CD57 <sup>+</sup>	2.5 ± 2	59 ± 14	.0001
CD8α <sup>+</sup>	30 ± 10	36 ± 12	.49
<b>CD3<sup>+</sup>low Perforin<sup>+</sup> NK cells</b>			
CD16 <sup>+</sup>	91 ± 2		
CD11b <sup>+</sup>	100	ND	
CD57 <sup>+</sup>	2.5 ± 2		
CD8α <sup>+</sup>	38 ± 10		
<b>CD3<sup>+</sup>αβ Perforin<sup>+</sup> CD8<sup>+</sup> T cells</b>			
CD28 <sup>-</sup>	10 ± 2	70 ± 23	.0004
CD11b <sup>+</sup>	13 ± 1	60 ± 17	.0001
CD57 <sup>+</sup>	1 ± 1	65 ± 20	.0001

\* The data represent mean ± SD of 19 experiments.

† The data represent mean ± SD of 20 experiments.

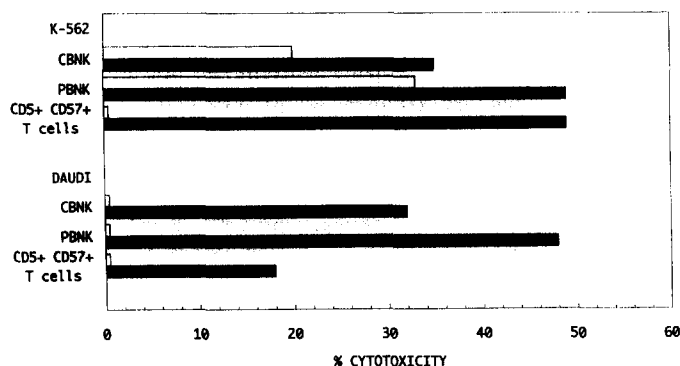
forin antisense oligonucleotides provided experimental evidence of an important role for this protein in the killing mechanism of infected or allogeneic grafted cells.<sup>11</sup> However, perforin does not constitute the only lytic pathway used by cytotoxic cells. As recently shown, a Fas-dependent killing mechanism also represents a major pathway of T-cell-mediated cytotoxicity.<sup>12,13</sup>

In this study, perforin was found to be constitutively expressed at birth in unstimulated CBLs. However, perforin expression was mainly restricted to two distinct NK-cell subsets. The first one, composed of mature NK cells, was well represented in CB. When compared with adult NK cells, these cells showed some significant differences, such as lack of CD57 and decreased CD56 expression, that might contribute to a decreased target-cell binding found in another study.<sup>14</sup> On the other hand, newborn CB large granular lymphocytes have been shown to contain fewer and less prominent granules than adult cells.<sup>15-17</sup> If a critical intracellular level of perforin is required by cytotoxic effectors to induce target cell lysis,<sup>18</sup> a decreased perforin content in unstimulated CB NK cells might favor a reduced spontaneous cytotoxic ability, as reported in most studies.<sup>14,16,17,19,20</sup> However,

we and others<sup>17</sup> observed a rapid and dramatic increase of NK-cell cytolytic efficiency after IL-2 activation. Moreover, we described for the first time that the CD56<sup>bright</sup> NK-cell subset, which represents about 10% of NK cells, lacked perforin in CB, just as in adult PB. These cells are known to show a low constitutive activity against NK-sensitive targets, and they can hardly perform any ADCC.<sup>21</sup> They might represent either an immature stage of NK-cell differentiation<sup>22,23</sup> or lymphokine-activated killer cell precursors.<sup>24</sup>

The second cell subset was composed of CD3<sup>low</sup> NK cells. Cell surface analysis showed the lack of T-cell-specific markers including TcR, CD5, and CD28. Moreover, this subset expressed only cCD3 proteins that have been described in pre-T cells<sup>25</sup> and immature NK cells.<sup>26</sup> The CD16 Ag, found in the majority of adult NK cells, was, in fact, expressed by greater than 90% of these cells. As reported by Phillips et al<sup>27</sup> and Lanier et al,<sup>28</sup> a small percentage of newborn CB NK cells expressed CD3ε, CD3δ, and CD3ζ cytoplasmic Ags, as did the majority of embryonic and fetal human NK cells.<sup>26</sup> Therefore, cCD3<sup>low</sup> CBLs expressing perforin might represent CB fetal-type or immature NK cells. Sanchez et al<sup>29</sup> found that 70% of fetal NK cells of 13- to 24-week gestation constitutively expressed the CD28 Ag, but all CB fetal-type perforin-positive NK cells that we examined were CD28<sup>-</sup>. Fetal NK cells were capable of both spontaneous major histocompatibility complex (MHC) non-restricted cytotoxicity and ADCC.<sup>27</sup>

Perforin expression in T lymphocytes was then investigated. In CBs, very few perforin-positive CD8<sup>+</sup> T lymphocytes could be detected (1% ± 0.5%). BM CD8<sup>+</sup> T cells were perforin-negative. In adult blood, a constitutive expression of perforin was found in a substantial proportion of the CD8 T-cell subset (30% ± 17%), although some variations in the number of CD8<sup>+</sup> T cells expressing perforin was pointed out in this study as well as in the work of Yagita et al.<sup>30</sup> These investigators further suggested that such cells might represent in vivo effector cytotoxic T lymphocytes sensitized by various Ags. Particular attention was paid in our study to examining the CD28 Ag expression on these cells. CD28 is the membrane receptor that specifically binds to B7 expressed on Ag-presenting cells.<sup>10</sup> Perforin-expressing T lymphocytes could be divided in CD28<sup>+</sup> and CD28<sup>-</sup> cells. CD28<sup>-</sup> cells represented 70% ± 23% of perforin-positive



**Fig 2. Cytotoxic activity of perforin-expressing cells in CB and adult PB.** CB NK cells (CBNK) and adult PB NK cells (PBNK) were purified as described in Materials and Methods. T cells were stained with PE-conjugated anti-CD5 and with FITC-conjugated anti-CD57. CD5<sup>+</sup> CD57<sup>+</sup> cells were separated by flow cytometry. Purified cell populations were tested for cytotoxic activity against <sup>51</sup>Cr-labeled K-562 target cells and the Daudi cell line, in the presence of PHA (PHA-P; final dilution, 1/600). The E:T ratio was 40:1. Results are shown for resting and 48-hour activated cells.

CD8<sup>+</sup>T cells in adults. We investigated the cytotoxic activity of CD28<sup>-</sup> CD8<sup>+</sup> perforin-positive T cells. They showed substantial lectin-dependent NK-like cytotoxicity against K-562 targets only after IL-2 activation. It was also shown that these cells generated moderate lymphokine-activated killer activity against the Daudi cell line. Moreover, Azuma et al<sup>31</sup> recently reported that CD28<sup>-</sup> CD8<sup>+</sup> T lymphocytes were capable of potent anti-CD3–induced cytotoxicity function without in vitro activation. It was striking to notice that, despite their distinct lineage, NK cells and CD8<sup>+</sup> perforin-positive T cells present in adult blood had similar phenotypical and functional features; they, indeed, shared several membrane receptor Ags from the integrin family, both lacked CD28 Ag expression, and they mediated MHC nonrestricted cytotoxic activity. CD8<sup>+</sup> T lymphocytes that constitutively express perforin, without coexpression of activation markers, might develop after in vivo Ag activation. This could be also the case for CD4<sup>+</sup> and  $\gamma\delta$ -T cells expressing perforin that are absent from CB but present in low numbers in adult PB.

As expected, granzyme B expression did not occur either in cord or in adult blood; analysis was performed using unstimulated lymphocytes, and it is generally admitted that granzyme B expression is only induced under T-cell activation.<sup>32</sup>

In conclusion, we investigated the cytolytic potential of CBLs as compared with adult lymphocytes, according to perforin and granzyme B expression. Neonatal NK cells expressed perforin and could efficiently lyse NK-sensitive target cells. The vast majority of CB TcR- $\alpha\beta$ <sup>+</sup>-T cells and all  $\gamma\delta$ <sup>+</sup>-T cells lacked this protein. Therefore, the spontaneous cytolytic function of CB cells appeared to be dependent on perforin-positive mature and immature NK cells. In adults, a subpopulation of TcR- $\alpha\beta$ <sup>+</sup> CD8<sup>+</sup> T cells lacking CD28 Ag had a constitutive expression of perforin, and, similar to NK cells, they were capable of MHC nonrestricted cytolytic function. The origin and development of these cells, absent from CB and BM but, in fact, detected in adult PB, still remains to be determined.

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