Human Fetal Liver-Derived CD7+CD2lowCD3−CD56− Clones That Express CD3γ, δ, and ε and Proliferate in Response to Interleukin-2 (IL-2), IL-3, IL-4, or IL-7: Implications for the Relationship Between T and Natural Killer Cells

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Cells from fetal liver or fetal and adult bone marrow that are membrane (m)CD3 negative and have not rearranged TCR genes but express CD3 proteins in their cytoplasm are considered to be committed T-cell progenitors. Recent findings question whether CD3 is T-cell specific because fetal natural killer (NK) cells have been shown to express cCD3 and ε proteins. To further examine the relationship between T and NK cells, we generated mCD3−cCD3+ clones from fetal liver. Two stable clones, FL412 and FL508, isolated from different donors, were selected on the basis of absence of the NK cell marker CD56. These clones shared a common phenotype of CD7+CD2lowCD3−CD4−CD8−CD5−CD6−CD11b+CD16−. Like fetal NK clones, these clones expressed cytoplasmic CD3δ and ε transcripts and proteins. However, the clones exhibited no or very low levels of cytotoxic activity against K562, JY, or Daudi, which were lysed efficiently by fetal NK clones. TCRγ, γ, and δ genes in these clones were in germline configuration. Furthermore, both FL412 and FL508 responded not only to interleukin-2 (IL-2), IL-4, or IL-7, but also to IL-3 with proliferation. These results suggest that FL412 and FL508 retained some characteristics of a putative T or NK precursor in the fetal liver and may be useful for analyses of this poorly defined cell type.

It has been established that T cells develop in the thymus from fetal liver or bone marrow-derived hematopoietic precursor cells. Hematopoietic stem cells first appear within the yolk sac in an early embryonic stage. During middle to late gestational stages, the sites of primary hematopoiesis change to the fetal liver and then to the bone marrow as hematopoietic stem cells presumably migrate. In mice, highly enriched hematopoietic stem cells from bone marrow or fetal liver have been shown not only to rescue lethally irradiated mice, but also to generate donor-derived T-cell colonies when injected intrathymically. Whereas all other hematopoietic cell lineages can be generated within fetal liver or bone marrow, the thymic microenvironment is essential for efficient differentiation and maturation of T-lineage cells. Despite recent progress, little is known about whether hematopoietic stem cells, common lymphoid stem cells, or committed T-cell precursors emigrate to colonize the thymus in physiologic situations.

Our knowledge of human T-cell precursors has been based on immunohistologic studies and tissue culture experiments. Previous studies have indicated that CD7+ is the only known T-cell lineage marker expressed on fetal hematopoietic cells before thymic colonization by T-cell precursors. These CD7+CD45+CD2−CD3− cells, which express cytoplasmic (c)CD3 but no TCR proteins, are present in 7-week fetal yolk sac, thorax/neck, and liver and may represent T-cell precursors.

One approach to further investigate CD7+mCD3−cCD3+ fetal liver cells is to establish clones of the relevant phenotype from fetal liver. We previously reported that mCD3−CD16− clones could be isolated from fetal liver and expanded in the presence of a feeder cell mixture of irradiated peripheral blood lymphocytes (PBL), irradiated JY cells, and phytohemagglutinin (PHA). These clones expressed cytoplasmic CD3δ and ε proteins, suggesting that they represented T-cell precursors. However, most of these clones shared a number of characteristics with natural killer (NK) cells, as they expressed high levels of CD56 and were able to mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity. More recently, we have found that freshly isolated mCD3−CD56−CD7+ NK cells from fetal liver express cytoplasmic CD3δ and ε transcripts and proteins. These latter Ag were also found to be expressed in NK cells from fetal spleen and in a proportion of NK cells from cord blood, but not in NK cells from adult peripheral blood mononuclear cells (PBMC). These findings suggested that NK and T cells may share a common precursor.

To further delineate the relationship between NK and T cells it was necessary to identify clones that share characteristics of these two cell types but are nevertheless distinct from functionally mature fetal T and NK cells. In this study we have established cloned lines of a novel cell type from fetal liver. The origin of these clones and possible implications for early T- and NK-cell development are discussed.

MATERIALS AND METHODS

Cell preparations. Human fetal liver tissue was obtained from elective therapeutic abortions of 12 to 22 weeks gestational age. Fetal liver samples were first minced into 2- to 3-mm cubes in 5 to 10 mL Yssel's medium containing 1% human serum. These tissue fragments were then gently pressed through a stainless steel mesh to produce single cell suspensions.

Monoclonal antibodies (MoAbs). MoAbs Leu-6 (CD1), Leu-5 (CD2), Leu-4 (CD3e), Leu-3 (CD4), Leu-1 (CD5), Leu-9 (CD7), Leu-2 (CD8), Leu-11 (CD16), Leu-19 (CD56), and Leu-7 (CD57) were purchased from Becton Dickinson (Sunnyvale, CA). MoAbs SPV-14 (CD6), BMA031 (TCRαβ), BF1 (TCRγ chain) (obtained from T Cell Sciences, Boston, MA), and TCRβ1 (anti-TCRβ chain)11 have been described previously. MoAb SP64, which recognizes the δ chain of CD3 (CD3δ), was a kind gift of Dr Cox Terhorst (Beth Israel Hospital, Boston MA). The other MoAbs...
used in this study are 9.3 (CD28), Q5/13 (HLA class I), BB10 (anti-p55-interleukin-2 [IL-2] receptor, a kind gift of Dr T. Wijdenes, Besancon, France) and 2RB (anti-p55-IL-2 receptor),14 which was a gift of Dr T. Uchiyama (Kyoto University, Kyoto, Japan).

**Cytokines.** Human recombinant (r)IL-2, rIL-4, and recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) were produced and purified at DNAX Research Institute. Units of IL-2 and IL-4 were defined as described.15 Human rIL-7 was purchased from R&D Systems, Inc (Minneapolis, MN). Human rIL-7 was obtained from Cos-7 cells transfected with a plasmid containing a full-length IL-7 cDNA, as previously described.16 The control used in this study was mock supernatant obtained from Cos-7 cells transfected with the same vector plasmid without cDNA insert.

**Cell surface and cytoplasmic phenotype analysis.** Cells were incubated on ice for 30 minutes with MoAbs in phosphate-buffered saline (PBS) containing 10 mg/mL bovine serum albumin (BSA) and 0.2 mg/mL NaNO₃, washed twice, and then incubated with fluorescein isothiocyanate-conjugated (Fab')₂ fragments of goat anti-mouse IgG (Tago, Burlingame, CA). After two washes, cells were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

Cyttoplasmic Ag were detected by the method described previously.17 Briefly, after fixation with 0.1% paraformaldehyde, cells were treated with 0.1% Triton X-100, incubated with anti-CD3 or anti-TCR MoAbs. As control we included IgGl myeloma proteins (Sigma, St Louis, MO) (SP-64, Leu-4, BF1, and TCR 61 are all IgGl). The cells were washed and incubated with FITC-conjugated (Fab')₂ fragments of goat anti-mouse IgG. The samples were analyzed on the FACScan.

**Cloning of CD3⁺CD8⁻CD56⁺ cells from fetal liver.** Freshly isolated fetal liver cells were cultured with phytoerythrin (PE)-labeled Leu-9 (anti-CD7) and FITC-labeled antLCD56 and the FITC/PE positive cells were cloned using a cloning device coupled to the FACStar plus as described previously.7

**Cloning of CD2⁻CD3⁻ fetal liver cells.** Fresh fetal liver cells were first cultured at 2 x 10⁵/mL with 1 x 10⁵ irradiated PBL/mL and 0.1 mg/mL PHA (Wellcome, Dartford, Kent, UK) in Yssel’s medium.4 After 1 week, 20 U/mL IL-2 was added to the culture. After 2 weeks, cells were stained with FITC-Leu-5 (anti-CD2) (Becton Dickinson) and PE-conjugated Leu-4 (anti-CD3). The CD2⁻CD3⁻ subpopulation was isolated by using a FACStar plus (Becton Dickinson). CD2⁻CD3⁻ cells were cloned by limiting dilution in 96-well round-bottom plates (Costar) in the presence of a feeder cell mixture of irradiated 5 x 10⁵ irradiated PBL/mL, 5 x 10⁵ irradiated JY cells/mL (an Epstein-Barr virus [EBV]-transformed B-cell line) and 0.05 mg/mL PHA. After 1 week, 100 mL of fresh medium containing 20 U/mL IL-2 was added to each well. After 2 weeks, clones were transferred to 24-well plates with the feeder cell mixture and further expanded with IL-2.

**Cytotoxicity assay.** Cytotoxicity was assayed according to the standard ⁵¹Cr release method as previously described.18 Briefly, 2,000 ⁵¹Cr-labeled target cells were mixed with effector cells in Yssel’s medium containing 1% human serum in 96-well round-bottom plates (Flow Laboratories, McLean, VA). The plates were centrifuged at 50g for 5 minutes and incubated for 4 hours at 37°C. The samples were harvested with a Skatron harvester (Lier, Norway) and counted in a gamma counter.

**Northern blot analysis.** Expression of CD3γ, 8, and e mRNA was examined by Northern blot analysis. The probes used in this study are the 900-bp XhoI fragment of pJ6 (CD3γ), the 900-bp XhoI fragment of pJ6 (CD3γ), the 1,200-bp PstI fragment of pAI (β-actin). Total RNA isolation, gel electrophoresis, RNA transfer, and hybridization were performed as described previously.17,19

**Southern blot analysis.** Southern blot analysis was performed to detect rearrangements of the TCR6γ, γ, and δ chain genes using the CB2 probe (400-bp BglII fragment of JUR-b2), the Jα1 probe PH60 (840-bp EcoRl fragment of mpCI7R9), and the Jβ1 probe (1,500-bp SacI fragment of Jαδ61), respectively. Isolation of high molecular DNA, digestion with restriction enzymes, gel electrophoresis, DNA transfer, and hybridization were performed as described previously.17,19

**Proliferation assay.** Proliferative responses of fetal liver-derived clones to various cytokines were measured by [³H]thymidine incorporation. Briefly, 2 x 10⁵ cells of fetal liver clones, harvested 5 days after coculture with the feeder cell mixture without exogenous IL-2, were plated in a total volume of 200 mL of Yssel’s medium containing 1% human serum. Serial dilutions of IL-2, IL-3, IL-4, IL-7, and GM-CSF were added to the responder cells (triplicates for each condition) in 96-well round-bottom plates (Flow Laboratories). The cells were pulsed with 1 mCi/well [³H]thymidine (Amersham, Arlington Heights, NJ) for the last 6 hours of a 72-hour incubation and harvested on glass-fiber filters. The radioactivity was counted with a liquid scintillation counter.

**Immunoprecipitation and Western blot analysis.** For immunoprecipitation, cells were lysed in 10 mmol/L Tris, 150 mmol/L NaCl, pH 7.2 containing 1% NP-40, 1 mL PMSF, and 20 Kallikrein inhibitor units (KU)/mL aprotinin for 20 minutes on ice or in 20 mmol/L triethanolamine, 150 mmol/L NaCl, 0.02% NaNO₃ buffer, pH 7.8, containing 0.12% Triton X-100 and 1% digitonin20 for 45 minutes on ice. Nuclei were removed by centrifugation (13,000g for 5 minutes). Cell lysates were preclarified three times with 10 mg packed Pansorbin (Calbiochem-Behring, San Diego, CA) coated with saturating amounts of rabbit antimonus Ig serum. Antigens were immunoprecipitated using Pansorbin coated with saturating amounts of control rabbit Ig, specific rabbit antiserum, or rabbit antimonus Ig and murine MoAb, as described.21 Immunoprecipitates were electrophoresed using 12% acrylamide gels. Proteins were visualized by autoradiography.

For Western blot analysis, cells were washed in PBS and then lysed at 1 x 10⁶ cells/mL in Tris-buffered saline (TBS, 50 mmol/L Tris, 150 mmol/L NaCl, pH 8.0) containing 1% NP-40, 20 KU/mL aprotinin (Sigma), 10 mmol/L iodoacetamide, and 1 mL PMSF (Sigma).21 After incubation for 20 minutes on ice, nuclei were pelleted by centrifugation (13,000g) for 5 minutes and lysates were added to an equal volume of 2 x Laemmli sample buffer22 with or without 10% 2-mercaptoethanol. Samples were incubated in a boiling water bath for 5 minutes. Twenty microfilters of cell lysate containing 10⁵ cell equivalents were electrophoresed using 12% acrylamide gels (BioRad, Richmond, CA) and transferred to Immobilon membranes (Millipore, Bedford, MA) using 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol transfer buffer. Membranes were incubated 2 hours overnight at 4°C on an orbital shaker platform in 100 mL blot buffer (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.02% NaNO₃, 0.05% Tween-20) containing 5% nonfat dry milk. Membranes were then incubated in 25 mL blot buffer containing 5% nonfat dry milk and 1/1,000 dilution of anti-CD3γ peptide antisum for 2 hours at room temperature on an orbital shaker platform. Rabbit anti-CD3γ antisum was generated against a peptide (GSIGKNHLV-KVYDYQEDGSVLLC) corresponding to the first 23 amino acids of the NH₂ terminus of the extracellular domain of the polypeptide (generously provided by Dr Art Weiss, UCSF, San Francisco, CA).22 After washing four times for 5 minutes in 100 mL blot buffer, membranes were incubated for 1 hour at room temperature in 25 mL blot buffer containing 0.5 mCi/mL [¹²⁵I] protein A (Amersham). Membranes were washing four times for 5 minutes in 100 mL blot buffer rinsed, in distilled water, and autoradiographed.
RESULTS

Isolation of CD2low CD3- clones from fetal liver. The objective of this study was to establish cloned lines of fetal liver cells that potentially represented T-cell progenitors. Because CD3 has previously been considered to be specific for the T-cell lineage, earlier attempts to isolate T-cell progenitors had focused on obtaining clones expressing CD3 in the cytoplasm but not on the membrane. However, recently we have found that mCD3-CD56+ fetal NK cells and cloned lines derived from these cells express CD3 transcripts and proteins in the cytoplasm. These results indicate that expression of CD3 proteins is not confined to T cells. Therefore, we attempted to isolate mCD3- lines from fetal liver that lacked NK cell characteristics. Unfractioned human fetal liver mononuclear cells were cultured with irradiated allogeneic PBL and PHA. In these cultures CD3+ cells became the predominant population, probably because of an outgrowth of mature T cells. However, about 20% to 40% of the cells were CD2-CD3-. Reasoning that the latter subset might contain an immature type of cells, these cells were isolated by fluorescence-activated cell sorting (FACS) and cloned by limiting dilution in the presence of a feeder cell mixture of irradiated PBL, irradiated JY cells (an EBV-transformed B-cell line), and PHA. The frequency of the clones that could be expanded was, in all experiments, 0.5% or lower. Clones were then screened for expression of CD2, CD3, and the NK marker CD56. All NK cells have been reported to express CD5624,25 and most of them are CD2+. Therefore, clones were sought that did not express CD56 and were negative or expressed low levels of CD2. Most CD2low clones expressed high levels of CD56 and were excluded for study. However, in four different cloning experiments we were successful in establishing five CD2lowCD56- clones. Two of these CD56- clones, FL412 and FL508, derived from 14-week and 18-week fetal liver, could be expanded sufficiently to permit analysis of function and antigenic phenotypes.

Figure 1 shows that clone FL508 is CD7+CD2low but does not express any other T-cell-specific markers including CD1, CD3, CD4, and CD8. Although not shown, CD5, CD6, and CD28 were also negative. Of the NK markers, CD16 was not detected and only a small proportion of FL508 cells weakly expressed CD56. They expressed CD7, CD11b, HLA class II, and IL-2 receptors (both p55 and p75). FL412 had essentially the same phenotype as FL508 (not shown).

Expression of CD3. T-cell progenitors are believed to express CD3. Therefore, expression of CD3 and CD3 expression of CD3 was explored by Northern blot analysis. For comparison, a mature mCD3+ T-cell clone, a representative fetal liver-derived mCD3+ cCD3+CD56+ NK clone (FL708) and a PBL-derived CD16-CD56+ NK clone were included (Fig 2). β-Actin mRNA was detected in all clones, which confirmed that equal amounts of RNA were loaded on the gel. It has been reported that fetal NK cells express CD3ε and δ while adult NK cells express only CD3ε mRNA. In accordance with this, the adult NK clone expressed only CD3ε mRNA, whereas a mature T-cell clone and fetal liver-derived clones FL508, FL412, and the fetal NK clone FL708 all expressed CD3ε and ε mRNA. Interestingly, CD3γ mRNA was detected in the mature T-cell clone, FL412 and FL508 but could hardly be detected in the fetal-liver-derived NK clone FL708. The amounts of CD3γ mRNA expressed in FL412 and FL508 were comparable with that of mature T-cell clone.

We next examined whether the two clones expressed CD3 and ε antigens and TCRβ and δ polypeptide chains. As shown in Fig 3, both FL412 and FL508 expressed...
cytoplasmic CD3\(\gamma\) and \(\epsilon\) but not TCR\(\beta\) or \(\delta\) polypeptide chains, as determined by immunofluorescent staining of fixed, permeabilized cells. The amounts of CD3\(\delta\) and \(\epsilon\) were comparable with those of Molt-4, a thymic leukemia line used as a positive control. The EBV-transformed B-cell line JY expressed none of these cytoplasmic Ag. Unfortunately, no CD3\(\gamma\) specific antibody was available that was suitable for cytoplasmic staining. Therefore, we analyzed expression of CD3\(\gamma\) protein in clone FL412 by Western blotting using a rabbit anti-CD3\(\gamma\) antibody raised against a peptide corresponding to the first 23 amino acids of the NH\(_2\) terminus. Digitonin lysates were prepared from FL412 and immunoprecipitated with anti-Leu-4 (anti-CD3\(\epsilon\)). After electrophoresis the immunoprecipitates were transferred to PVDF membranes and immunoblotted with the anti-CD3\(\gamma\) peptide serum. As shown in Fig 4, lane 2, the CD3\(\epsilon\) precipitate reacted strongly with the anti-CD3\(\gamma\) serum, indicating the presence of CD3\(\epsilon,\gamma\) complexes in clone FL412. In addition, CD3\(\gamma\) was detected after blotting of whole cell lysates by Western blotting with the anti-CD3\(\gamma\) serum. Three bands were observed with relative mobilities of 28, 24, and 18 Kd, presumably representing mature (28 Kd) and immature (24 and 18 Kd) forms of CD3\(\gamma\), respectively, as described previously. Many fetal CD56\(^+\) NK clones do not express CD3\(\gamma\) transcripts at all, but in a proportion of these clones such transcripts were detectable by PCR analysis (not shown). Blotting with anti-CD3\(\gamma\) of CD3\(\epsilon\) precipitates or of whole cell lysates of fetal NK clones that did express CD3\(\gamma\) transcripts showed the presence of only some CD3\(\gamma\) proteins in fetal NK clones. No CD3\(\gamma\) proteins could be detected in lysates of clones that did not have CD3\(\gamma\) transcripts as determined by PCR (not shown).

**Southern blot analysis.** Rearrangement of the TCR genes is initiated in the thymus by as yet unknown signals. T-cell progenitors present in the fetal liver are assumed to have their TCR genes in germline configuration. To examine the status of the TCR genes in FL412 and FL508, Southern blot analysis was performed with DNA samples of clones FL412 and FL508. Hybridization with the C\(\beta2\) probe showed a 22.6-kb band in BamHI digests and 7.4- and 3.4-kb bands in HindIII digests in these clones as well as JY cells (the germline control) (Fig 5A). Similarly, hybridization with the J\(\gamma1\) probe showed 3.2- and 1.5-kb bands in EcoRI digests and 16- and 9-kb bands in KpnI digests (Fig 5B). Hybridization with the J\(\beta1\) probe showed 6.4- and 5.5-kb bands in EcoRI digests and a 16-kb band in KpnI digests of genomic DNA from JY cells, FL412, and FL508 (Fig 5C). DNA from a mature T-cell clone that was included as a positive control gave deleted patterns (Fig 5, lane d). These results indicate that the TCR\(\beta\), \(\gamma\), and \(\delta\) genes of FL412 and FL508 are in germline configuration.

**Cytotoxic activities of clones FL412 and FL508.** To further examine a possible relationship between FL412, FL508, and NK cells, cytotoxic activities were measured against target cells known to be highly sensitive to functional fetal and adult NK clones. As shown in Table 1, FL508 and FL412 exhibited no or very low levels of cytotoxicity against K562, JY, or Daudi compared with fetal liver-derived CD3\(^-\)CD16\(^-\)CD56\(\text{high}\) clones. Together with the low expression of CD56, these findings indicate that FL412 and FL508 are different from functionally mature fetal NK cells.

**Proliferative response to cytokines.** FL412 and FL508, which are not transformed, require coculture and restimulation with the feeder cell mixture at intervals of no longer than 4 weeks and subsequent addition of IL-2 for growth. To define their responsiveness to various cytokines, these clones were cultured with IL-2, IL-3, IL-4, or IL-7 for 72 hours and \(^{3}H\)thymidine incorporation for the last 6 hours was measured. The proliferative responses of FL508 and FL412 were compared with those of CD56\(\text{high}\) fetal NK clones and two adult NK clones. As shown in Table 2, all clones responded to IL-2. Most clones responded also to IL-4, but with one exception, the NK clones responded weakly if at all. Clones FL508 and FL412 responded to IL-3...
and IL-7, while the NK clones did not respond to these cytokines. The responses of FL508 and FL412 to IL-3, IL-4, and IL-7 were modest, but these clones responded in a dose-dependent way (data not shown). To determine whether proliferative response to IL-3 or IL-7 is mediated by IL-2, we examined the effect of anti-IL-2R MoAb in these proliferation experiments. As shown in Fig 6, anti-IL-2R MoAb blocked almost 90% of the IL-2 response. In contrast, the IL-7 response was hardly affected and the IL-3 response was only partially (30%) blocked. Our findings do not exclude that some of the effects are indirect, but they indicate that the proliferative responses are not dependent on IL-2.

**DISCUSSION**

It has been shown that CD7+CD3+mCD3− cells are present in fetal liver and in bone marrow. It was believed that CD7+CD3+ fetal liver cells represent T-cell precursors. However, recent findings question whether cCD3 expression is restricted to the T-cell lineage, as freshly isolated fetal liver NK cells were found to express cCD3, suggesting that T and NK cells are more closely related than was previously appreciated. Circumstantial evidence has suggested the possibility that T cells and NK cells may share a common differentiation pathway. Both cell lineages express of a
number of cell surface Ag not found on mature B cells or myeloid cells, ie, CD2, CD7, and CD8a. Moreover, some adult T cells express the NK marker CD56, and this antigen can be induced on T cells in tissue culture conditions.10 The transmembrane form of CD16, which is found on the majority (but not on all)32 adult NK cells, is expressed on a minority of T cells.33 Both T and NK cells express CD3e mRNA.36 In addition, NK clones have been shown to express germline transcripts for TCRβ37 and TCRγ.38 There are also functional similarities between T and NK cells. Interferon-γ is produced by T cells, cloned T-cell lines,29 and NK cells,32 but not by other cell types. Furthermore, like NK cells, activated TCRβ+ T cells40 and TCRγδ+ cells41,42 can mediate MHC nonrestricted cytotoxic activities.

The purpose of the present study was to establish and characterize cloned lines of mCD3- fetal liver cells that, like putative T-cell progenitors, express cytoplasmic CD3, but lack characteristics of functionally mature NK cells. Such cell lines may be useful to better delineate the relationship between T and NK cells. We describe here the establishment of a novel type of clones, FL412 and FL508, from human fetal liver that are distinct from fetal NK cells in several aspects. Like T-cell progenitors29 clones FL412 and FL508 expressed low levels of CD2. This does not distinguish these clones from functional fetal NK clones because variable expression of CD2 on fetal NK clones from fetal liver has also been observed (J.H.P., unpublished data, 1991). However, clones FL412 and FL508 were CD16- and, perhaps more importantly, expressed only very low levels of CD56. CD56, which is expressed on CD16+2425 and CD16- NK cells26 is highly expressed on virtually every

| Table 1. Cytotoxic Activities of Clones FL412, FL508, and Fetal NK Clones Against Different Target Cells |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | K562*                           | Daudi*                          | JY*                            |
| Clones                          | 30:11                           | 10:1                            | 3:1                            | 30:1                            | 10:1                            | 3:1                            | 30:1                            | 10:1                            | 3:1                            |
| FL412                           | 7                               | 2                               | 1                              | 2                               | 0                               | 0                              | 11                              | 7                               | 2                               |
| FL508                           | 11                              | 10                              | 5                              | 4                               | 2                               | 2                              | 17                              | 8                               | 8                               |
| FL708                           | NT                              | 72                              | 45                             | NT                              | 64                              | 45                             | 63                              | 30                              | 15                              |
| FL739                           | NT                              | 74                              | 46                             | NT                              | 75                              | 51                             | 55                              | 38                              | 11                              |
| Abbreviation: NT, not tested.   | *Target cells.                  | †Effector-to-target cell ratio.  |                                |                                 |                                 |                                 |                                 |                                 |                                 |

*Values represent cpm sample – cpm of cells in medium only. Standard deviations were always <10%.
†Clones FL708, 734, 739, B1.13, and 182.S1 are fetal CD56+NK clones. 16+7 and 16+11 are CD16+ and CD16− adult NK clones, respectively.
‡A representative of three experiments is shown.
functional NK clone of fetal and adult origin we have analyzed so far (H.S. and J.H.P., unpublished data, 1991). CD56 is negative on freshly isolated fetal liver T cells (J.H.P., unpublished data, 1991). This Ag can be found on some cultured CD3+ T-cell clones from adult PBMC and also on fetal liver mCD3+ T-cell clones (results not shown). Therefore, the presence of CD56 on cloned fetal cells cultured in vitro is not exclusively confined to NK cells, but the absence of CD56 indicates that FL508 and FL412 are not mature NK cells. This notion is further supported by the finding that FL412 and FL508 displayed minimal cytotoxic activities against target cells that are lysed strongly by NK clones. In addition, differences in cytokine responsiveness of some TCRαβ+ T cells that do not express CD4 or CD8 can be observed during NK differentiation. The finding that some NK clones express CD3e and δ, but not γ, may suggest that CD3γ is downregulated before δ. However, verification of this notion requires a direct comparison of the proportions of CD3e+δ+ and CD3e+δ+ fetal liver NK cells by cytoplasmic staining. Unfortunately, anti-CD3γ MoAbs suitable for cytoplasmic staining are not available. It should also be noted that the hypothesis that CD3 proteins are downregulated in an ordered way when NK development proceeds requires the assumption that fetal and adult NK cells represent cells within the same developmental pathway. It is, alternatively, possible that CD3δ+ eNK cells and NK cells that express only CD3e transcripts represent distinct subsets of NK cells. Fetal type NK cells expressing CD3e and high levels of ε may be replaced by adult type mRNA CD3ε+ NK cells after completion of embryogenesis, a situation comparable with the “waves” of TCRγδ+ cells that can be observed during T-cell ontogeny. Further studies are required to solve this issue.

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