

# Ontogeny of Human Natural Killer (NK) Cells: Fetal NK Cells Mediate Cytolytic Function and Express Cytoplasmic CD3 $\epsilon$ , $\delta$ Proteins

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## Summary

Natural killer (NK) cells have been defined as CD3 $\epsilon$ <sup>-</sup>,CD16<sup>+</sup> and/or CD56<sup>+</sup> lymphocytes that mediate major histocompatibility complex (MHC)-unrestricted cytotoxicity against certain tumors and virus-infected cells. Unlike T lymphocytes, NK cells do not rearrange or productively express T cell antigen receptor genes. Moreover, NK cells from adults have been reported to not express CD3 $\gamma$ ,  $\delta$ , or  $\epsilon$  proteins on the cell surface or in the cytoplasm. Nonetheless, NK cells have been shown to share a number of antigenic and functional similarities to T cells that suggest the possibility of common origins. In this report, we demonstrate that functional NK cells exist in liver at early stages of human embryonic development. Freshly isolated fetal NK cells mediated MHC-unrestricted cytotoxicity against NK-sensitive targets and acquired the ability to lyse NK-resistant tumors after overnight culture in interleukin 2. Unlike adult NK cells, freshly isolated fetal liver NK cells and clones derived from these cells, as well as a subset of cord blood NK cells, express substantial levels of CD3 $\delta$  and CD3 $\epsilon$  proteins in the cytoplasm. Expression of CD3 $\epsilon$  and CD3 $\delta$  transcripts and cytoplasmic proteins in fetal NK clones was confirmed by polymerase chain reaction and Western blot analysis. These findings support the concept that NK and T cells may arise from a common progenitor that expresses components of the CD3 complex. Alternatively, it is possible that the cytoplasmic CD3 $\delta$ , $\epsilon$ <sup>+</sup> fetal NK cells represent a distinct subpopulation of NK cells that is predominant in the fetus, but replaced by the cytoplasmic CD3 $\delta$ , $\epsilon$ <sup>-</sup> adult NK cell population after embryogenesis.

NK cells<sup>1</sup> are a subpopulation of lymphocytes arising from bone marrow progenitor cells that are distinct from the progenitors of T and B cells (1). NK cells have the capacity to kill certain tumors and virus-infected cells, but unlike CTL, do not require the presence of MHC class I or II antigens to recognize their targets. In addition to mediating cytolytic function, activated NK cells produce regulatory cytokines and growth factors, including IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$  (2). The receptors on the membrane of NK cells that are responsible for recognition of tumors or virus-infected targets are presently unknown. NK cells do not rearrange TCR genes and do not express CD3 $\epsilon$  on the cell surface (3–8). Human NK cells can be identified by the phenotype membrane CD3 $\epsilon$ <sup>-</sup> (mCD3 $\epsilon$ ) and CD16<sup>+</sup> and/or CD56<sup>+</sup> (9). Mature NK cells have been reported to not ex-

press CD3 $\gamma$ , $\delta$ , $\epsilon$  proteins, but do express CD3 $\zeta$  in association with Fc $\gamma$ RIII (CD16) (10, 11).

Although TCR rearrangement clearly distinguishes mature NK and T lymphocytes, it is possible that these cells arise from a common progenitor before the rearrangement events that commit cells to the T lineage. While there has been extensive analysis of T lymphocyte development, particularly in the thymus, there has been essentially no information concerning NK cell ontogeny or differentiation. In this study, we have documented the existence and characteristics of functional NK cells present in human embryonic and fetal hematopoietic tissues.

## Materials and Methods

**Preparation of Lymphocytes.** Embryonic and fetal tissues were obtained from elective therapeutic abortions. Gestation age was determined by crown-rump length and ranged from 6 to 24 wk. Mononuclear cells from fetal liver, spleen, and thymus were prepared by standard Ficoll-Hypaque procedures and cells suspended

<sup>1</sup> Throughout this article, the term NK cell will be used to designate lymphocytes with the antigenic phenotype (mCD3 $\epsilon$ <sup>-</sup>,CD56<sup>+</sup>), which includes both CD16<sup>-</sup> and CD16<sup>+</sup> NK cell subsets.

in complete medium (RPMI 1640 [M. A. Bioproducts, Walkersville, MD], 10% FCS [JR Scientific, Woodland, CA], 1 mM L-glutamine, and 1% penicillin-streptomycin). Fetal tissues were obtained and used with the approval of the Committee for the Protection of Human Subjects at our institutes. Adult peripheral blood was obtained from the Stanford Blood Center (Palo Alto, CA). Mononuclear cells from cord blood of full-term newborns and peripheral blood of healthy adults were isolated by standard Ficoll-Hypaque techniques. Monocytes and B cells were depleted by adherence to plastic and nylon wool, and large low buoyant density cells were isolated by centrifugation through discontinuous gradients of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), as previously described (12).

**mAbs.** mAbs against the following antigens were used: CD1 (anti-Leu-6); CD2 (anti-Leu-5b); CD3 $\epsilon$  (anti-Leu-4); CD3 $\delta$  CD3 $\delta$  (mAb Sp64 [13], generously provided by Dr. C. Terhorst, Harvard University, Boston, MA); TCR- $\beta$  ( $\beta$ F1; T Cell Sciences, Cambridge, MA); TCR- $\delta$  ( $\delta$ -TCS1; T Cell Sciences); TCR- $\alpha$  ( $\alpha$ F1; T Cell Sciences); CD3 $\zeta$  (TIA-2 [14], generously provided by Dr. P. Anderson, Dana Farber Cancer Institute, Boston, MA); CD4 (anti-Leu-3a); CD5 (anti-Leu-1); CD6 (MB-G6, American Type Culture Collection [ATCC], Rockville, MD); CD7 (anti-Leu-9); CD8 (anti-Leu-2b); CD10 (anti-Calla); CD11a (L7); CD11b (anti-Leu-15); CD11c (anti-Leu-M5); CD14 (anti-Leu-M3); CD15 (anti-Leu-M1); CD16 (anti-Leu-11a); CD18 (anti-LFA-1 $\beta$ ); CD19 (anti-Leu-12); CD25 (anti-IL-2R- $\alpha$ , BD); CD33 (anti-Leu-M9); CD34 (QBend/10.4.19; AMAC, Westbrook, ME); CD38 (anti-Leu-17); CD44 (anti-Leu-44); CD45 (anti-common leukocyte antigen); CD45RA (anti-Leu-18); CD45RO (UCHL-1); CD56 (anti-Leu-19 and L185); CD57 (anti-Leu-7); CD69 (anti-Leu-23); CD71 (anti-transferrin receptor); glycophorin A (10F7Mn, ATCC); p75 IL-2R protein (TU-27 [15], generously provided by Dr. K. Sugamura, Sendai, Japan); Leu-8 (anti-Leu 8); HLA-DR (anti-HLA-DR, BD); and TdT (anti-TdT; Supertechs, Bethesda, MD). All mAbs were generously provided by Becton Dickinson Immunocytometry Systems (San Jose, CA), unless otherwise noted. Fluorochrome-conjugated rat anti-mouse Ig isotype-specific mAbs were generously provided by Mr. David Buck (Becton Dickinson Immunocytometry Systems).

**Immunofluorescence and Flow Cytometry.** Cells were stained with fluorochrome-conjugated mAb for detection of cell surface antigens by immunofluorescence, as described (16). Appropriate-purified or fluorochrome-conjugated isotype-matched control Igs were used in all experiments. Samples were analyzed using a FACScan<sup>®</sup> (Becton Dickinson & Co.).

For detection of cytoplasmic CD3 and TCR proteins in fresh fetal NK cells, cells were first stained with an IgM isotype anti-CD56 mAb (clone L185), followed by a PE-conjugated rat anti-mouse IgM isotype-specific mAb. The cells were fixed for 10 min in ice-cold acetone, rehydrated for 20 min in PBS, and washed twice in wash buffer (PBS, pH 7.3, with 0.05% sodium azide and 2% FCS). Fixed cells were stained for 30 min with optimal amounts of purified mAb to detect cytoplasmic antigens, washed twice in wash buffer, and then stained for an additional 30 min with optimal amounts of FITC-conjugated antibodies specific for the isotypes of the mAbs used for the detection of the cytoplasmic antigens (anti-mouse IgG1 and anti-mouse IgG2).

For the detection of TdT, viable cells stained with PE anti-CD56 (as described above) were fixed for 30 min at 4°C in 1% paraformaldehyde PBS (pH 7.3) and then incubated an additional 5 min in 0.1% (final concentration) triton X-100 (Sigma Chemical Co., St. Louis, MO). The fixed cells were washed twice in wash buffer and then stained with FITC-conjugated anti-terminal deox-

ynucleotidyl transferase (TdT) according to manufacturer's recommendations.

**Purification of NK Cells.** NK cells were isolated either by positive selection using FACS<sup>®</sup> or by negative depletion of other cell types using antibody-coated magnetic beads. For cell sorting experiments, cells were stained with a PE-conjugated anti-CD56 mAb and an FITC-conjugated anti-CD3 $\epsilon$  mAb, and mCD3 $\epsilon$ <sup>-</sup>, CD56<sup>+</sup> lymphocytes were isolated to >98% purity using a FACStar Plus<sup>®</sup> flow cytometer (Becton Dickinson & Co.), as previously described (12). For magnetic bead depletion, cells suspended in PBS containing 0.1% sodium azide were stained for 20 min on ice with saturating quantities of anti-CD3, CD4, CD5, CD14, CD19, CD33, CD71, and glycophorin A mAbs (all of which had been predetermined not to react with the cell surface of fetal NK cells). After staining, cells were washed extensively and combined with magnetic beads coated with sheep anti-mouse IgG (Dynal Inc., Great Neck, NY), according to manufacturer's recommendation.

NK cells were cloned at one cell per well using a single cell deposition system of the FACStar Plus<sup>®</sup> flow cytometer. Clones were established using culture conditions described previously (17), and cloning efficiencies were generally 10–20%. All clones generated by this procedure were mCD3 $\epsilon$ <sup>-</sup>, CD56<sup>+</sup> and mediated NK cytotoxic function.

**Cell-mediated Cytotoxicity.** Cytotoxic activity was measured in a standard 4-h <sup>51</sup>Cr radioisotope release assay (18). NK-sensitive targets assayed were K562 (erythroleukemia) and Jurkat (T leukemia). NK-resistant targets were Colo-205 (colon carcinoma), Colo-320 (colon carcinoma), JY (EBV-transformed B lymphoblastoid cell), SB (EBV-transformed B lymphoblastoid cell), Ramos (Burkitt's lymphoma), Arent (EBV-transformed B lymphoblastoid cell), Raji (Burkitt's lymphoma), and RPMI 8866 (myeloma). All tumor cell lines were obtained from ATCC, except Arent, which was generously provided by Dr. Ed Engleman (Stanford University). Antibody-redirected killing assays using the FCR-bearing mouse P815 tumor cell line were performed as previously described (19).

**PCR.** RNA was isolated as described by Chirgwin et al. (20), with minor modifications. cDNA was prepared in a 40- $\mu$ l reaction volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA, 0.1  $\mu$ g/ml BSA, 1 mM dNTPs, 2 mM spermidine, 70 U RNasin (Promega Biotec, Madison, WI), 0.4 mM Na pyrophosphate, 10 U AMV reverse transcriptase (Pharmacia Fine Chemicals, Piscataway, NJ), and 10  $\mu$ g of total RNA. The reaction mixture was incubated at 42°C. After 45 min, RNA was hydrolyzed by adding 40  $\mu$ l 0.3 N NaOH and 30 mM EDTA, and boiling for 5 min. cDNA was phenol/chloroform extracted and ethanol precipitated. PCR were performed (43 cycles at 94°C for 1 min; 55°C for 1 min; 72°C for 1 min) using a GeneAmp Kit (Perkin-Elmer Cetus, Norwalk, CT). Primers for PCR were: CD3 $\epsilon$ , 5'AGTTGGCGTTTGGGGGCAAGATGGT-AATGAAGAAA3' and 5'CCCAGGAAACAGGGAGTCGCAGG-GGGACTGAGAG3'; CD3 $\delta$ , 5'TGCAATACCGCATCACATG-GGTAGAGGGAACGGT3' and 5'CTTGTTCCGAGCCCAAGTTT-CCTCCAAGGTGGCTGT3'; CD3 $\gamma$ , 5'AACCACTCCAAGTGT-ATTACAGAATGTGTCAGAAC3' and 5'ATTCCTCCTCAACTG-GTTTCCTTGAAGGTGGCTGT3'; CD3 $\zeta$ , 5'TGGAAGGCGCT-TTTCACCGCGGCCATCCTGCAGGC3' and 5'GCGAGGGGG-CAGGGCCTGCATGTGAAGGGCGTCGT3'; CD4, 5'GCTGAT-GCTGAGCTTGAAACTGGAG3' and 5'GCTACATGTCTTCTG-AAACCGGTGAG3';  $\beta_2$ -microglobulin, 5'TATAAGCTTCTTTC-TGGCCTTGAGGCT3' and 5'TATCTAGATGGAGCAACCTG-CTCAGA3'.

**Southern Blot Analysis.** Genomic DNA was isolated from NK clones, digested with restriction enzymes, transferred to nitrocel-

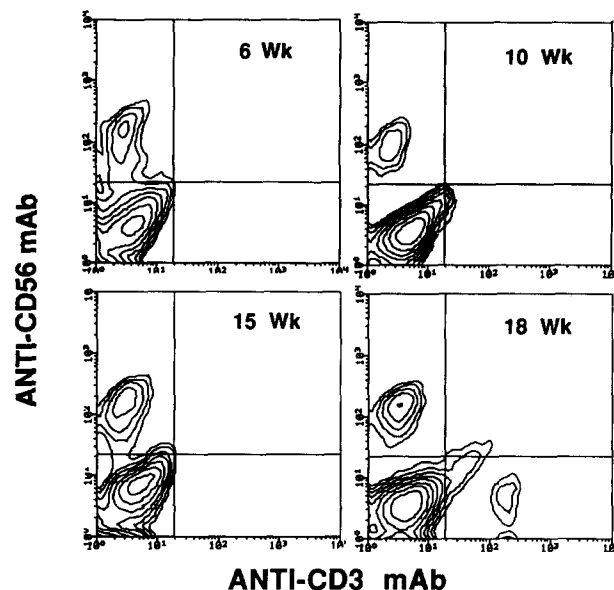
lulose membranes, and hybridized with  $^{32}\text{P}$ -labeled DNA probes, as described (21).

**Immunoprecipitation and Western Blot Analysis.** For Western blot analysis, cells were washed in PBS and then lysed at  $10^8$  cells/ml in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 8.0) containing 1% NP-40, 20 Kallikrein inhibitor U/ml aprotinin (Sigma Chemical Co.), and 1 mM PMSF (Sigma Chemical Co.) (22). After incubation for 20 min on ice, nuclei were pelleted by centrifugation (13,000 g) for 5 min, and lysates were added to an equal volume of  $2\times$  Laemmli sample buffer (23) containing 10% 2-ME. Samples were incubated in a boiling water bath for 5 min. 20  $\mu\text{l}$  of cell lysate containing  $10^6$  cell equivalents was subjected to electrophoresis using 12% acrylamide gels (Bio-Rad Laboratories, Richmond, CA) and transferred (100 V, 1 h) to Immobilon-P membranes (Millipore Continental Water Systems, Bedford, MA) using transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were incubated overnight at  $4^\circ\text{C}$  on a orbital shaker platform in 100 ml blot buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.02%  $\text{NaN}_3$ , 0.05% Tween-20) containing 5% nonfat dry milk. Membranes were then incubated in 25 ml blot buffer containing 5% nonfat dry milk and 5  $\mu\text{g}/\text{ml}$  affinity purified rabbit anti-CD3 $\epsilon$  peptide antibody or 1/500 dilution of anti-CD3 $\delta$  mAb SP64 for 2 h at room temperature on an orbital shaker platform. After washing four times for 5 min in 100 ml blot buffer, membranes were incubated for 1 h at room temperature in 25 ml blot buffer containing 0.5  $\mu\text{Ci}/\text{ml}$   $^{125}\text{I}$ -labeled protein A (for detection of rabbit antibody) or 0.2  $\mu\text{Ci}/\text{ml}$   $^{125}\text{I}$ -labeled sheep anti-mouse Ig (for detection of SP64 mAb) (Amersham Corp., Arlington Heights, IL). Membranes were washed four times for 5 min in 100 ml blot buffer, rinsed in distilled water, and autoradiographed. Affinity-purified rabbit anti-CD3 $\epsilon$  peptide antibody was purchased from Dako (Denmark) and was generated against amino acids 156–168 (RRGHHLFPTQTMSF) in the cytoplasmic domain of human CD3 $\epsilon$  (24).

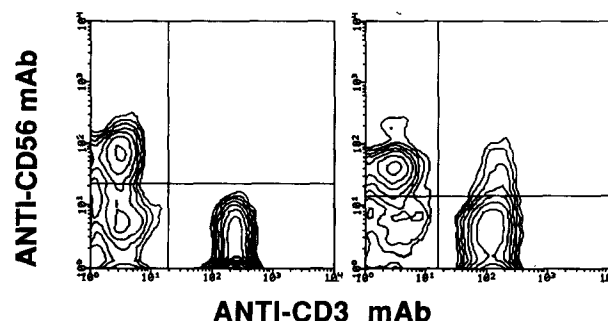
## Results

**Identification of NK Cells in Embryonic and Fetal Tissues.** Flow cytometric analysis was used to identify the presence and frequency of NK cells in embryonic and fetal tissues. Remarkably, a substantial proportion of the mononuclear cells isolated from livers of 6–24 wk gestation expressed the phenotype of adult NK cells (e.g., mCD3 $\epsilon^-$ , CD56 $^+$ ). As early as 6 wk gestation, 5–8% of the total mononuclear cells were mCD3 $\epsilon^-$ , CD56 $^+$ . Between 8 and 18 wk gestation, the total percentage of mCD3 $\epsilon^-$ , CD56 $^+$  cells gradually increased to 15–25%. Representative data are presented in Fig. 1 A. It should be noted that while we have previously reported the presence of a small subset of mCD3 $\epsilon^+$ , CD56 $^+$  T lymphocytes in adult blood (9), we never detected the presence of CD56 $^+$  T cells in any fetal tissues. mCD3 $\epsilon^+$ , CD56 $^-$  T lymphocytes were initially observed in fetal liver at  $\sim 15$ –16 wk gestation and comprised 1–2% of total liver cells. The proportion of T cells gradually increased through 18 wk gestation. Analysis of mononuclear cells from 20–24-wk fetal livers revealed equal percentages of NK and T cells based on flow cytometric analysis ( $\sim 15$ –20%; data not shown). NK cells were also identified in umbilical cord blood obtained at birth from healthy full-term neonates. Approximately 10–15% of

**A**



**B**



**Figure 1.** Antigenic phenotype of embryonic, fetal, and newborn NK cells. (A) Mononuclear cells were obtained from embryonic and fetal livers at various gestation ages and stained with PE-conjugated anti-CD56 and FITC-conjugated anti-CD3 $\epsilon$  mAb. Cells were analyzed by flow cytometry. Two-color contour maps (four-decade log scales) displaying FITC and PE fluorescence were divided into quadrants based on  $>95\%$  of the cells residing in the lower left quadrant after staining with isotype control mAbs. Of the total mononuclear liver cells, NK cells (mCD3 $\epsilon^-$ , CD56 $^+$ ) comprised 8% at 6 wk gestation, 11% at 10 wk gestation, 16% at 15 wk gestation, and 18% at 18 wk gestation. The small finger of low density double staining cells at a  $45^\circ$  angle from the lower left quadrant represent nonspecific staining and are present in isotype control tubes. (B) Low buoyant density lymphocytes were isolated from newborn cord blood and adult peripheral blood and stained as in A. Approximately 35% of the low buoyant density cells from newborn cord blood (left) and 40% from adult peripheral blood (right) were identified as NK cells (mCD3 $\epsilon^-$ , CD56 $^+$ ).

cord lymphocytes were NK cells, comparable with their frequency in adult blood. Moreover like adult NK cells, cord blood NK cells preferentially migrated with the low buoyant density cells on Percoll gradients where they comprise 35–60% of lymphocytes depending on the donor (Fig. 1 B). At least

three embryos or fetuses were examined for each gestational age shown.

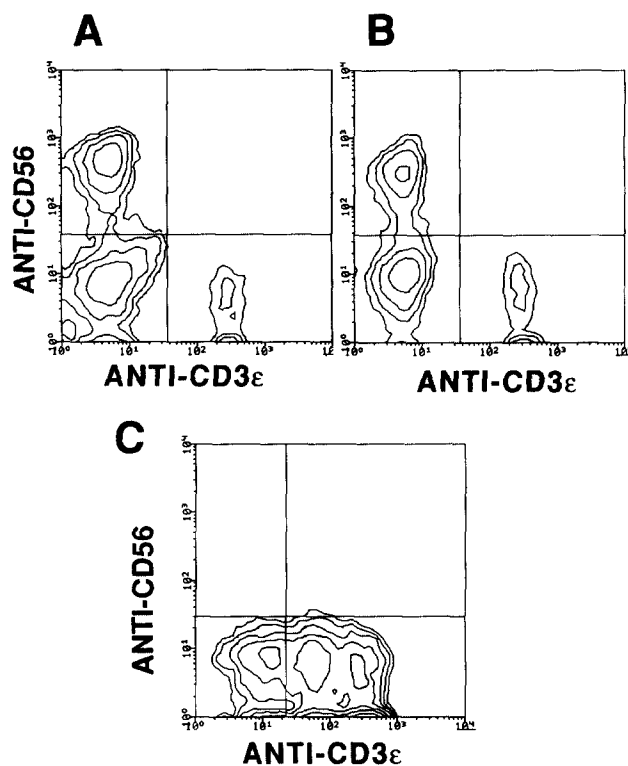
The total mononuclear cell population isolated from fetal livers consisted of enucleated erythrocytes and leukocytes, as well as other cell types of unknown origin. Therefore, to estimate the frequency of NK cells within the leukocyte population, we determined the frequency of NK cells within the population of cells expressing the common leukocyte antigen CD45. This was accomplished by simultaneous three-color immunofluorescence using PE-conjugated anti-CD56, FITC-conjugated anti-CD3 $\epsilon$ , and peridinin chlorophyll protein (PerCp)-conjugated anti-CD45. By this analysis, NK cells comprised from 35 to 70% of the CD45-positive leukocytes in embryonic/fetal livers of 6–24 wk gestation (Fig. 2). NK cells were also observed in fetal spleens between 15 and 24 wk gestation, where they comprised 15–30% of the CD45<sup>+</sup> cells (Fig. 2 B). NK cells were not readily observed in fetal thymus specimens between 16 and 24 wk gestation (Fig. 2 C).

**Freshly Isolated Fetal NK Cells Possess Spontaneous and IL-2-induced Cytolytic Function.** NK cells were purified from fetal liver (15–24 wk gestation), newborn cord blood, and adult peripheral blood by flow cytometry, and were assessed for cytolytic activity against NK-sensitive tumor cell targets (K562 and Jurkat) and an NK-resistant tumor cell target (Colo-205) in a 4-h radioisotope release assay. Freshly isolated fetal NK cells demonstrated significant cytolytic activity against NK-sensitive tumor cell targets (Fig. 3 A). However, the level of cytotoxicity of fetal NK cells was lower than that observed for newborn or adult NK cells (Fig. 3 B). In general, lytic activity of the purified liver NK cell populations increased with age of gestation.

Overnight incubation of fetal, newborn, or adult NK cells in rIL-2 (50 IU/ml) resulted in substantial augmentation of cytolytic activity (Fig. 3, C and D). Fetal liver NK cells derived from 15 and 18 wk gestation did not demonstrate the magnitude of cytotoxicity observed for 24-wk gestation NK cells or newborn NK cells, particularly against NK-resistant tumor cell targets.

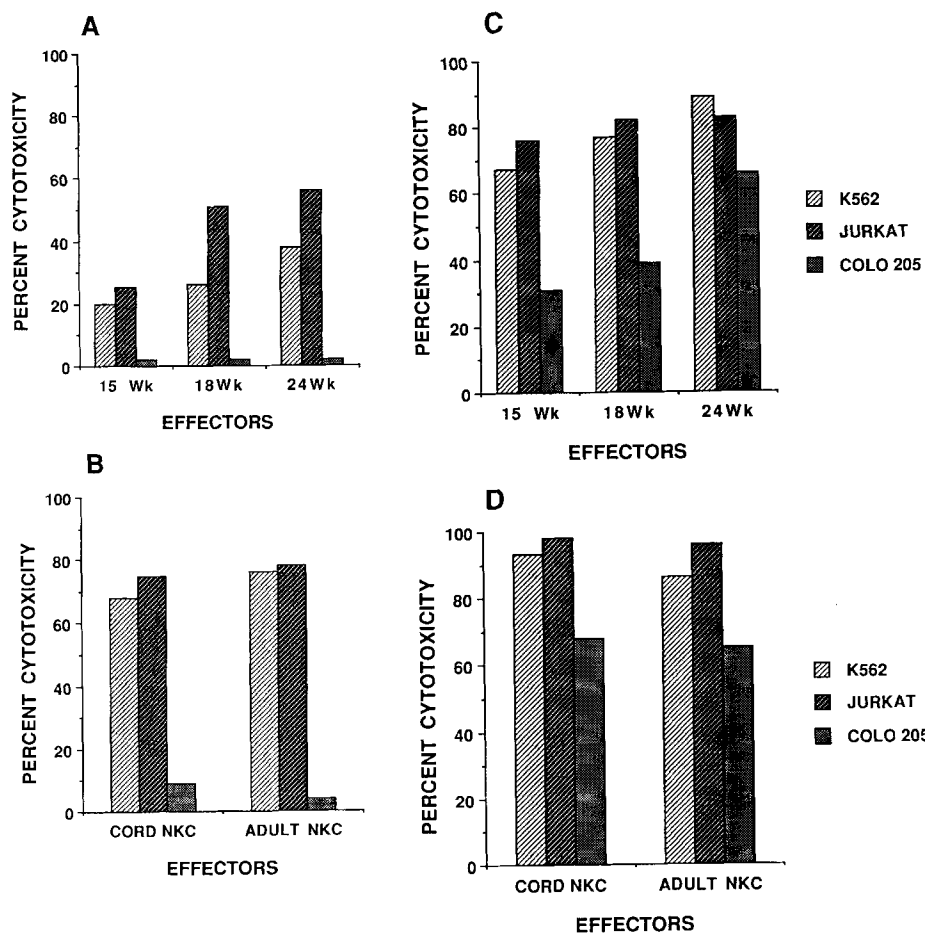
Since a substantial percentage of fetal NK cells expressed CD16 (Fc $\gamma$ RIII) at low levels on the cell surface (30–50% of total NK cells depending on the donor), assays were performed to determine if this molecule was competent to trigger cytotoxic activity. mAbs against CD16 have been shown to initiate cytotoxicity against Fc receptor-bearing NK-resistant targets (19). Purified fetal liver NK cells (18 wk gestation), newborn cord blood NK cells, and adult peripheral NK cells were combined with either a nonreactive isotype control mAb, anti-CD16 mAb, or anti-CD56 mAb, and incubated for 4 h at 37°C. Results from these experiments clearly showed that the CD16 molecule on fetal NK cells could efficiently signal cytotoxicity (Fig. 4). The magnitude of cytotoxicity was less for fetal NK cells than that observed for either newborn or adult NK cell populations; however, this may be accounted for by the lower percentage of fetal NK cells expressing CD16.

**Cytoplasmic Expression of CD3 Proteins.** Results from these studies have established that the freshly isolated mCD3 $\epsilon$ <sup>+</sup>,



**Figure 2.** Antigenic phenotype of NK cells in fetal liver, spleen, and thymus. Mononuclear cells were isolated from liver (A), spleen (B), and thymus (C) obtained from a 16 wk gestation fetus. Cells were stained and analyzed as described in Fig. 1. Approximately 38% of liver, 32% of spleen, and <1% of thymus cells were NK cells (mCD3 $\epsilon$ <sup>+</sup>, CD56<sup>+</sup>). Similar results were obtained from fetuses of 18 and 20 wk gestation.

CD56<sup>+</sup> lymphocyte population present in fetal hematopoietic tissues mediates spontaneous and IL-2-induced MHC-unrestricted cytotoxicity against tumor cell targets and possesses ADCC function. Adult NK cells have been previously reported to express CD3 $\zeta$  protein in association with CD16 (11, 25), but to lack membrane and cytoplasmic expression of CD3 $\epsilon$  proteins (26). To compare fetal with adult NK cells, freshly isolated fetal liver, fetal spleen, newborn cord blood, and adult peripheral blood NK cells were stained with PE-conjugated anti-CD56 mAb, fixed, and costained to detect the presence of cytoplasmic CD3 proteins. Consistent with previous studies, adult NK cells expressed cytoplasmic CD3 $\zeta$  protein and did not stain with anti-CD3 $\epsilon$  or anti-CD3 $\delta$  mAb (Fig. 5 C). Surprisingly, the majority (50–80%) of fetal NK cells showed prominent expression of CD3 $\epsilon$ , CD3 $\delta$ , and CD3 $\zeta$  proteins (Fig. 5 A). In the example shown in Fig. 5, careful examination of the fluorescence histograms of fetal NK cells stained for cytoplasmic CD3 $\epsilon$  revealed that a small proportion (~20%) lacked detectable CD3 $\epsilon$ , whereas the cytoplasmic CD3 $\epsilon$ -positive NK cells demonstrated two distinct levels of protein expression. Cytoplasmic CD3 $\epsilon$  proteins were detected in both the CD16<sup>+</sup> and CD16<sup>+</sup> subsets of fetal NK cells (data not shown). The majority of fetal NK cells also expressed detectable levels of cytoplasmic CD3 $\delta$  protein, in-



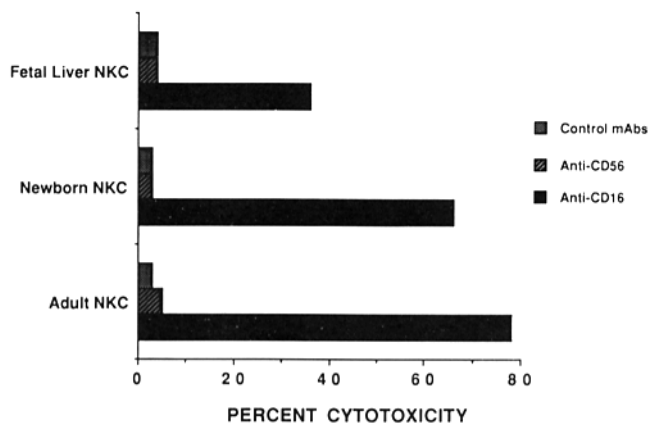
**Figure 3.** Freshly isolated fetal and newborn NK cells demonstrate spontaneous and IL-2-induced cytotoxicity. NK cells were purified from 15, 18, and 24 wk gestation fetal livers (A), newborn cord blood, and adult peripheral blood (B), and were assayed for cytotoxic activity. The same purified populations were also activated overnight in the presence of 50 U/ml of rIL-2 and then assayed for cytotoxic capabilities. E/T ratio was 25:1.

indicating that most must coexpress both CD3 $\delta$  and CD3 $\epsilon$ . In most fetal tissues examined, the proportion of cytoplasmic CD3 $\epsilon$ -positive fetal NK cells exceeded the frequency of CD3 $\delta$ -positive cells, suggesting the possibility that CD3 $\epsilon^+$ , $\delta^-$  NK cells may exist. As was observed for adult NK cells, essentially all fetal NK cells expressed cytoplasmic CD3 $\zeta$  protein. Analysis of cytoplasmic CD3 proteins in newborn cord blood NK cells also revealed the presence of a small proportion of NK cells expressing CD3 $\epsilon$  and CD3 $\delta$  proteins (Fig. 5 B). The percentage of newborn NK cells expressing CD3 $\epsilon$  and CD3 $\delta$  was extremely variable from donor to donor. Using mAbs against CD3 $\epsilon$  and CD3 $\delta$ , we have never observed cytoplasmic CD3 $\epsilon$  or CD3 $\delta$  in adult NK cells. mAb specific for CD3 $\gamma$  were not available to determine expression of this subunit in freshly isolated fetal tissues. Cytoplasmic staining with mAbs against framework determinants of TCR- $\alpha$ , TCR- $\beta$ , and TCR- $\delta$  failed to reveal these proteins in either fetal or adult NK cells (data not shown).

To determine if the CD3 proteins were stably expressed in fetal NK cells, long-term (>4 mo) clones of mCD3 $\epsilon^-$ , CD56 $^+$  fetal liver NK cells were established using a FACS<sup>®</sup> single cell cloning system. Over 100 individual fetal NK cell clones were established and analyzed for cytolytic function, cell surface antigenic phenotype, and the expression of cyto-

plasmic CD3 proteins. The majority of these clones (70%) expressed cytoplasmic CD3 $\epsilon$ , CD3 $\delta$ , and CD3 $\zeta$  proteins (Fig. 6 B). Approximately 10% of the fetal NK cell clones expressed CD3 $\epsilon$  and CD3 $\zeta$ , but no detectable CD3 $\delta$ . The remaining 20% of fetal NK clones expressed only CD3 $\zeta$  protein (Fig. 6 A), which was characteristic of all adult peripheral blood NK cell clones. Both CD16 $^-$  and CD16 $^+$  fetal NK cell clones have been generated, and cytoplasmic CD3 $\epsilon$  and  $\delta$  proteins are variably expressed in both types of NK cell clones (data not shown). Regardless of the expression of cytoplasmic CD3 proteins, all fetal NK clones were mCD3 $\epsilon^-$ , CD56 $^+$  and demonstrated a very similar spectrum of cytolytic activity against a broad panel of tumor cell targets (two representative clones are presented in Fig. 7).

**Analysis of TCR Rearrangement, CD3 Transcription, and CD3 Protein Expression in NK Clones.** Fetal NK clones were analyzed by PCR for the presence of transcripts of CD3 $\gamma$ , CD3 $\delta$ , and CD3 $\epsilon$ . Primers for  $\beta_2$ -microglobulin and CD4 were used as a positive and negative controls, respectively. Results from representative clones are shown in Fig. 8. All fetal NK clones examined expressed CD3 $\epsilon$  transcripts, consistent with prior observations using adult NK clones (26). Several fetal NK clones expressed CD3 $\delta$  transcripts, in accordance with and confirming the results from the cytoplasmic staining ex-



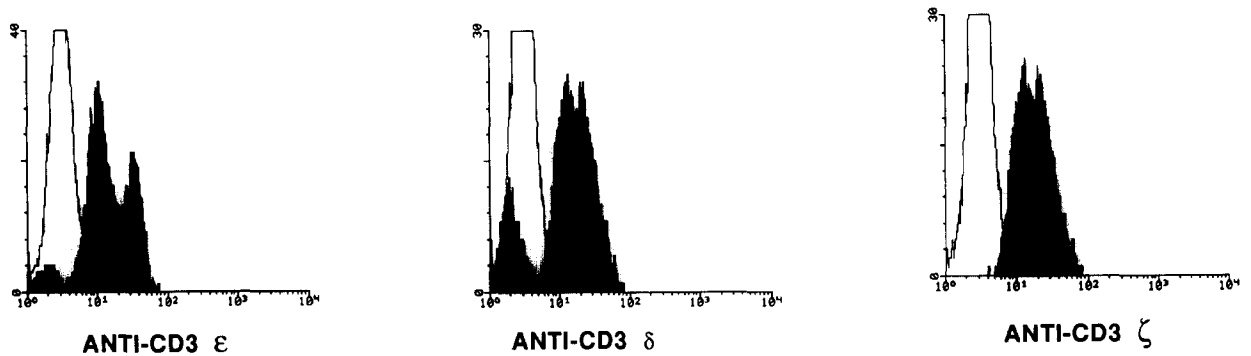
**Figure 4.** Freshly isolated fetal and newborn NK cells mediate anti-CD16-dependent redirected cytotoxicity. NK cells were purified from 18 wk gestation fetal liver, newborn cord blood, and adult peripheral blood by flow cytometry, and assayed for redirected cytotoxicity of P815 tumor cells in the presence of 10  $\mu$ g/ml of isotype control, anti-CD56, or anti-CD16 mAb.

periments. CD3 $\gamma$  transcripts were either absent or present in only very low levels in most fetal NK clones, including some clones that expressed high levels of cytoplasmic CD3 $\epsilon$  and CD3 $\delta$ . However, CD3 $\gamma$  transcripts were detected in certain fetal NK clones (e.g., NKB1.11). Representative fetal NK clones were analyzed for rearrangement of TCR- $\beta$  and TCR- $\gamma$  genes, and all were found to be in germline configuration (Fig. 9).

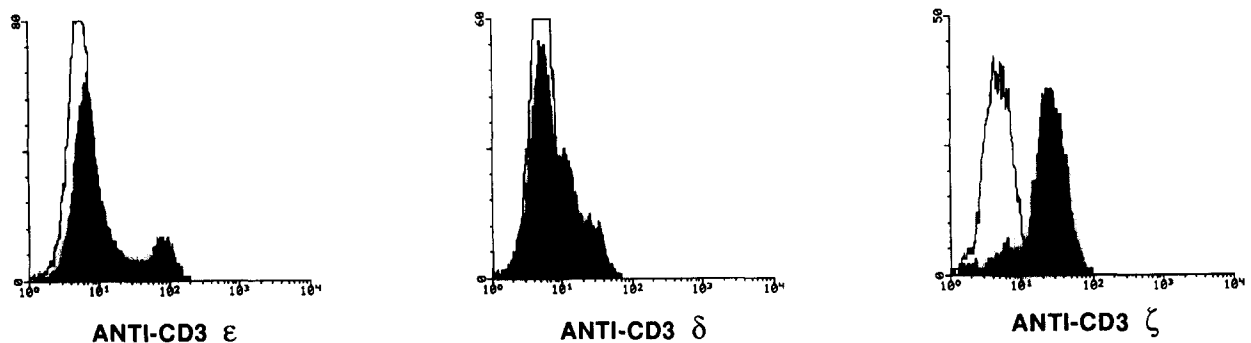
Western blot analysis was performed to confirm results from the PCR and immunofluorescence experiments. As shown in Fig. 10, the presence of CD3 $\epsilon$  and CD3 $\delta$  proteins of the appropriate molecular weight were detected in lysates of fetal NK clones NKB1.6, NKB1.13, NKB1.18, NKB1.11, and cord

Fetal NK cells were assayed at an E/T ratio of 25:1, while newborn and adult NK E:T was 12:1. NK cells were >95% pure based on reanalysis of the sorted NK cell populations.

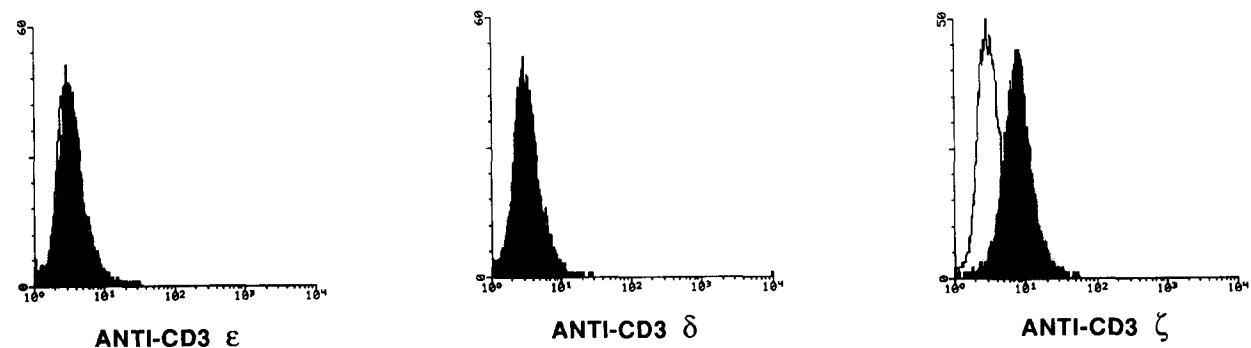
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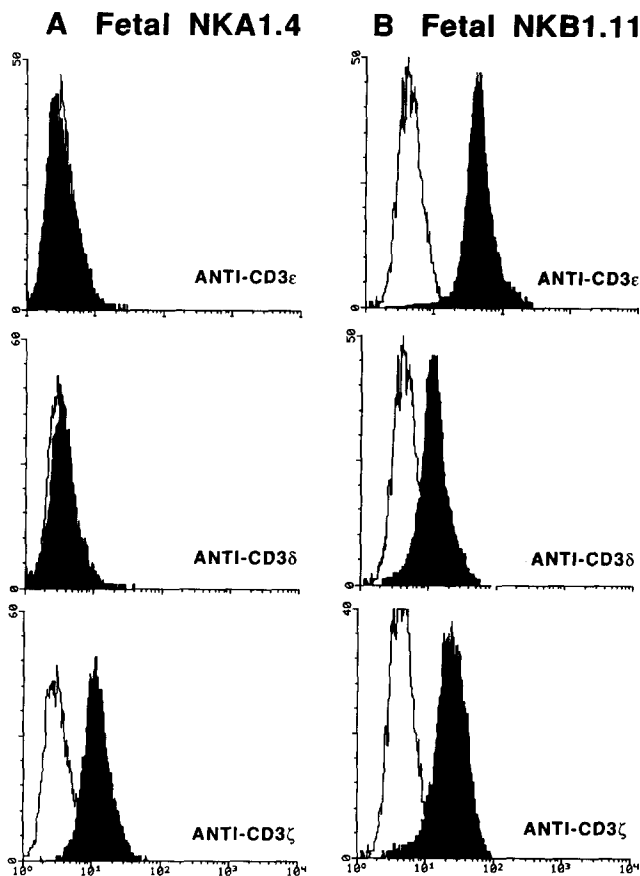


**B**



**C**

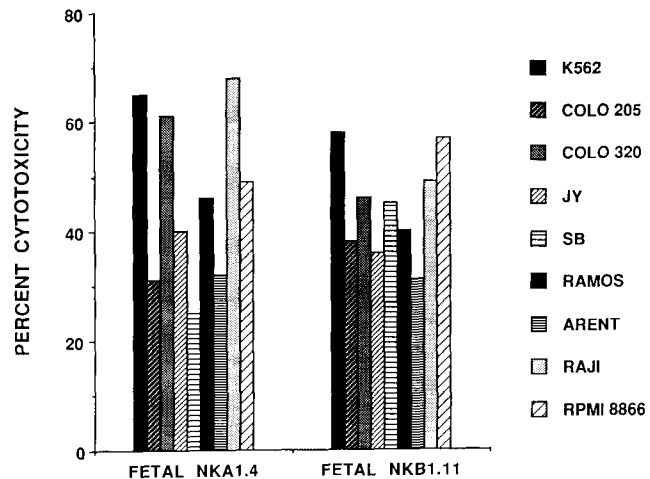




**Figure 6.** Expression of cytoplasmic CD3 proteins in fetal NK cell clones. mCD3ε<sup>+</sup>,CD56<sup>+</sup> fetal NK cell clones were established as described in Materials and Methods and maintained in culture for >4 mo. Analysis of CD3 protein expression is displayed for two representative clones. Fetal NK clone NKA1.4 (A) is representative of those clones expressing only cytoplasmic CD3ζ, which is typical for all NK cell clones derived from adult peripheral blood. Approximately 20% of fetal NK cell clones expressed only CD3ζ protein. The majority of fetal NK cell clones expressed cytoplasmic CD3ε, CD3δ, and CD3ζ proteins (B). Approximately 70% of fetal NK cell clones ( $n > 100$ ) expressed cytoplasmic CD3 proteins similar to clone NKB1.11.

blood NK clone CB.9. CD3 proteins were detected in Jurkat T leukemia cells (positive control), but not in Ramos or Raji Burkitt's lymphoma cells (negative controls).

**Comparative Studies of Antigenic Phenotypes of Fetal and Adult NK Cells.** Since the expression of cytoplasmic CD3ε,δ proteins in embryonic and fetal NK cells clearly distinguishes them from adult NK cells, we analyzed freshly isolated fetal and adult NK cells by three color immunofluorescence using a comprehensive panel of mAbs against other leukocyte differentiation antigens. Fetal liver mononuclear cells of 13–18 wk gestation were compared with low buoyant density cord

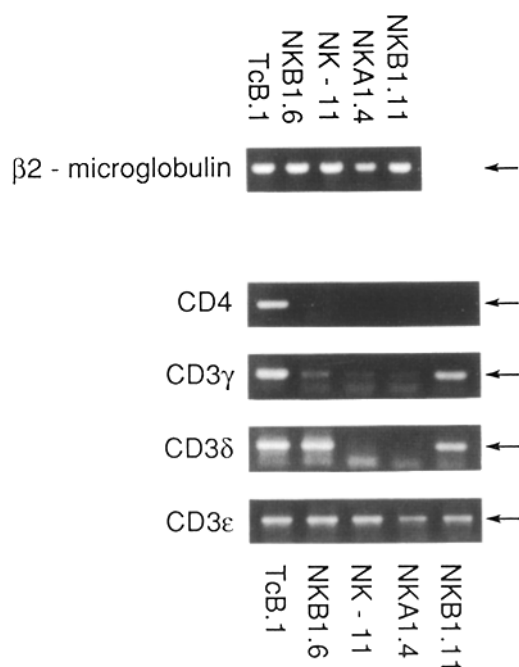


**Figure 7.** Cytotoxic activity of fetal NK cell clones. Fetal NK cell clones described in Fig. 4 were assayed for cytotoxic activity against a broad panel of tumor cell targets in a 4-h <sup>51</sup>Cr release assay. E/T was 3:1.

blood and adult PBL (enriched for NK cells). By placing an electronic gate on mCD3ε<sup>+</sup>,CD56<sup>+</sup> lymphocytes, it was shown that NK cells derived from fetal liver, cord blood, and adult peripheral blood displayed remarkable phenotypic similarities. NK cells, regardless of their source, did not express surface CD1, CD4, CD5, CD6, CD10, CD14, CD15, CD19, or CD45RO. Moreover, they did not express the hematopoietic progenitor antigen, CD34, nor did they express (TdT). Like adult NK cells, most fetal and cord blood NK cells expressed CD2, CD7, CD11a-c, CD18, CD38, CD45, and CD45RA, as well as the p75 IL-2R (data not shown). Consistent with prior reports, CD57 was not found on fetal or cord blood NK cells (27), but was detected on ~50% of adult NK cells (18). In contrast, the CD69 antigen, which has been characterized as an inducible lymphocyte activation antigen, was prominently expressed on all fetal NK cells and the majority of cord blood NK cells (Fig. 11).

Previously, we have reported that distinct subsets of adult peripheral blood NK cells can be identified based on the presence or absence of CD16 expression and the relative amount of CD56 (9, 28). Interestingly, fetal and newborn NK cells generally expressed slightly higher surface density of the CD56 antigen than was observed on the majority of adult peripheral blood NK cells (Fig. 1). Although CD16 was expressed on >90% of adult and newborn NK cells, fetal NK cells from either the liver or spleen generally expressed this antigen on ~30–50% of the cells (Fig. 11 A). The CD16 antigen on fetal NK cells was present in low surface density compared with the high surface expression of this antigen on newborn and adult NK cells. Phenotypic analysis of cord blood lym-

**Figure 5.** Expression of cytoplasmic CD3 proteins in freshly isolated NK cell populations. Mononuclear cells isolated from 16 wk gestation fetal liver (A) and cord blood low buoyant density cells (B), and adult peripheral blood low buoyant density cells (C) were surface stained with PE-conjugated anti-CD56 mAb, fixed, permeabilized, and stained for cytoplasmic CD3ε,δ, and ζ. By setting a gate on the CD56-positive cells, the percentage of cells expressing CD3 subunits was determined. In this fetal liver (A), ~85% of NK cells expressed cytoplasmic CD3ε, 60% expressed CD3δ, and 97% expressed CD3ζ proteins. Approximately 15% of newborn cord blood NK cells (B) showed cytoplasmic CD3ε, 30% CD3δ, and 95% CD3ζ protein. Adult peripheral blood NK cells (C) demonstrated only cytoplasmic CD3ζ.

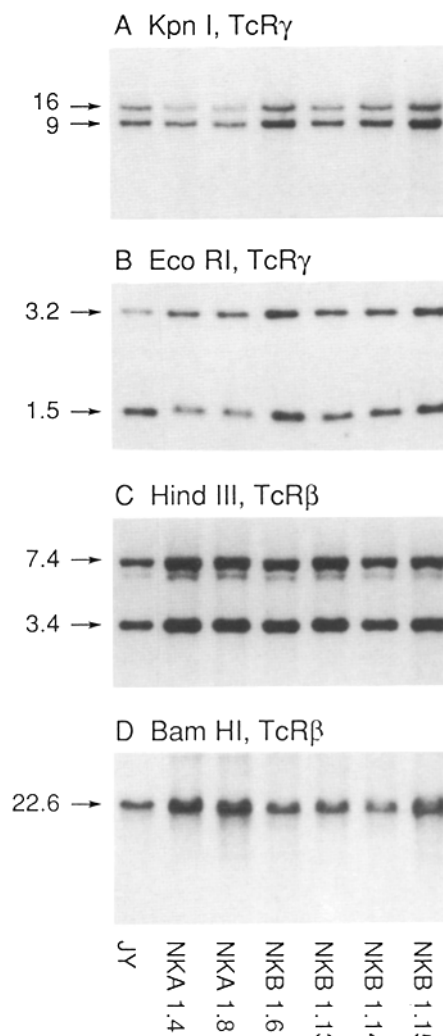


**Figure 8.** PCR analysis of CD3 transcription. RNA was isolated from a CD4<sup>+</sup> T cell clone (TcB.1; positive control), adult peripheral blood NK clone NK11, and fetal liver NK clones NK1.6, NKA1.4, and NKA1.11. cDNA was generated and expression of CD3ε, CD3δ, CD3γ, and CD4, and β<sub>2</sub>-microglobulin was assayed using appropriate primers. The arrow in each panel represents the expected size of the PCR product.

phocytes also revealed a significant population of mCD3ε<sup>-</sup> lymphocytes that expressed CD16, but did not express detectable CD56 antigen (Fig. 11 B). These cells were also identified in fetal liver between 16 and 24 wk gestation at very low frequencies (<1%). The relationship of these cells to NK cells is at present unknown and needs further investigation.

## Discussion

The antigenic and functional properties of mature NK cells from adults have been extensively investigated. Based on these studies, it is believed that NK cells are distinct from B and T cells and likely represent a third lymphoid lineage (29). However, the developmental relationship of B, T, and NK cells has not been delineated, and lymphoid progenitor cells have not been identified. Therefore, the primary objective of the present study was to carefully analyze the lymphoid populations of the developing human embryo and fetus for the presence of NK cells or possible NK progenitor populations. To our surprise, substantial numbers of NK cells were detected in all embryonic liver (6–9 wk gestation), fetal liver (9–23 wk gestation), fetal spleen, and cord blood populations studied. These NK cells were identified by the same criteria that have been used to define adult NK cells (e.g., mCD3ε<sup>-</sup>, CD56<sup>+</sup> lymphocytes), and like adult NK cells populations contained both CD16<sup>-</sup> and CD16<sup>+</sup> subsets. By

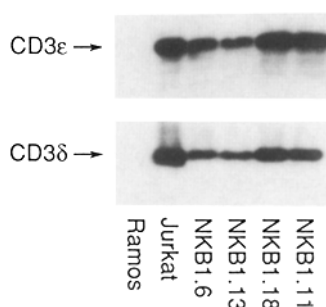


**Figure 9.** Southern blot analysis of fetal NK clones. High molecular weight DNA was isolated from JY (B lymphoblastoid cell line; germline control) and a panel of fetal NK clones. 15 μg DNA from each was digested with the indicated restriction enzyme and fragments were subjected to electrophoresis through 1% agarose gels. Membranes were hybridized with radiolabeled Cβ1 probe (41) or Jγ1 PH60 (42) probes.

contrast with these peripheral lymphoid tissues, a substantial proportion of NK cells was not observed in fetal or neonatal thymus (<<1%). Moreover, we have shown that freshly isolated embryonic, fetal, and cord blood NK cells mediated MHC-unrestricted cytotoxicity against NK-sensitive tumors and acquired the ability to lyse NK-resistant tumors after overnight culture with IL-2.

The antigenic and functional properties of embryonic and fetal NK cells are very similar to a small subset of adult NK cells we have previously described based on the phenotype mCD3ε<sup>-</sup>, CD56<sup>bright</sup> (9, 28). This population comprises <1% of PBL and was identified based on the observation that these cells expressed higher amounts of CD56 antigen and lower levels of CD16 than the majority of adult NK cells. Embryonic and fetal NK cells share a number of similarities





**Figure 10.** Western blot analysis of CD3 proteins. NP-40 detergent lysates were prepared from Ramos (Burkitt's lymphoma; negative control), Jurkat (T leukemia; positive control), and fetal liver NK clones.  $10^6$  cell equivalents of lysate were subjected to electrophoresis using 12% acrylamide gels and transferred to Immobilon membranes. Membranes were blotted with affinity-purified rabbit anti-CD3 $\epsilon$  peptide antibody or mouse anti-human CD3 $\delta$

mAb SP64, as indicated. Bound antibody was detected using  $^{125}$ I-labeled protein A or sheep anti-mouse Ig, respectively. Proteins of the predicted molecular weight of CD3 $\epsilon$  and CD3 $\delta$  are indicated by the arrow in each panel. Raji, another Burkitt's lymphoma used as a specificity control, was also negative (not shown).

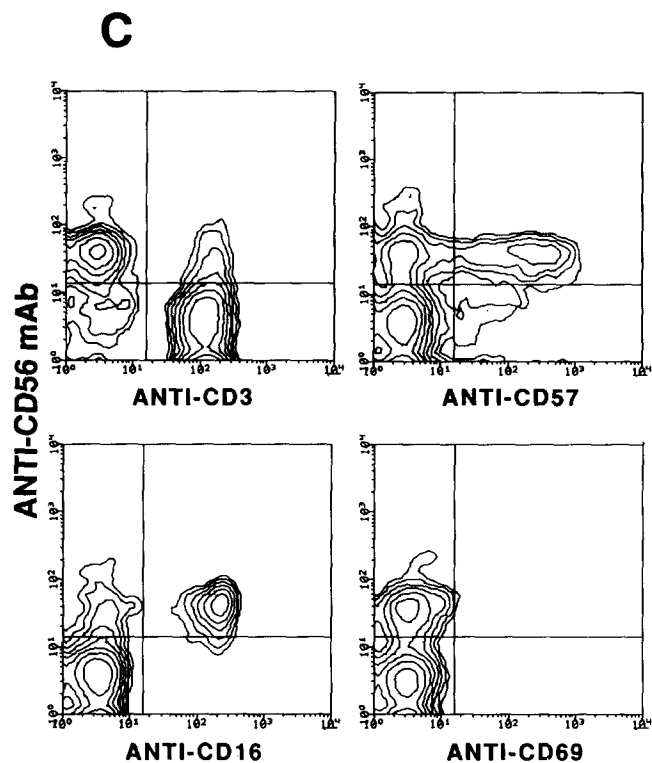
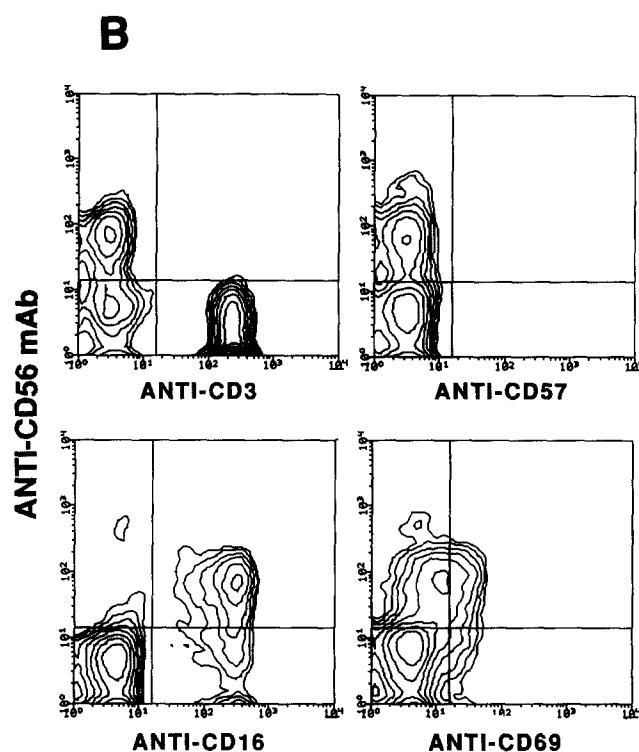
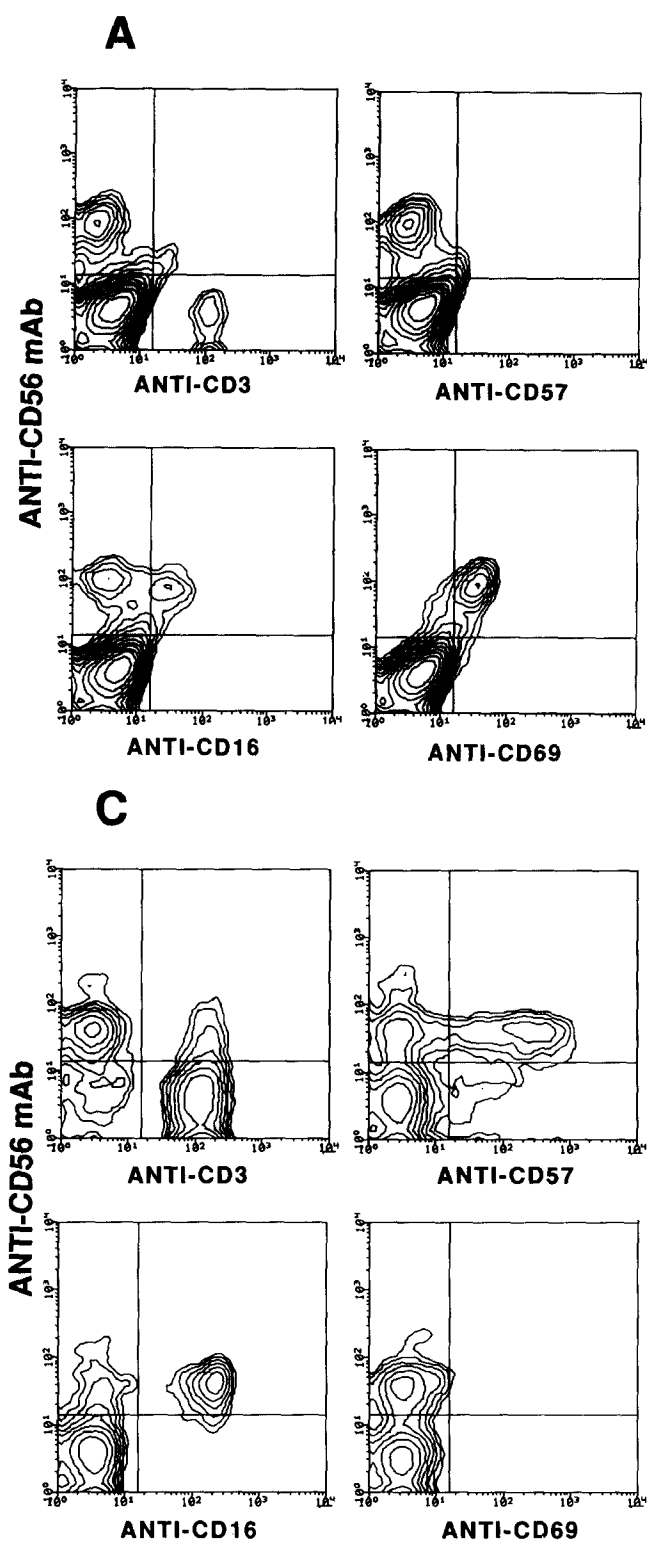
with the CD56<sup>bright</sup> NK cell subset in adult peripheral blood: (a) both express high surface density CD56 and lack or express only low levels of CD16; (b) neither express CD57; (c) both express high affinity IL-2Rs and respond to low concentrations of IL-2; and (d) both display lower constitutive cytolytic activity against NK-sensitive targets than the majority of circulating NK cells. We have previously proposed that the CD56<sup>bright</sup> NK cells may represent a more immature stage of NK cell differentiation (9, 28). The numerous similarities between fetal NK cells and adult peripheral blood CD56<sup>bright</sup> NK cells add support to this hypothesis; however, further studies are required to determine if they are related to a common differentiation pathway. However, an important distinction is that adult peripheral blood CD56<sup>bright</sup> NK cells do not express cytoplasmic CD3 $\epsilon$  or CD3 $\delta$ , but do express CD3 $\zeta$ , as determined by immunofluorescent staining using mAbs against these structures (J. Phillips and L. Lanier, unpublished observation).

While prior studies on the existence and function of NK cells in the human fetus have been contradictory and of limited scope (30–32), our present results clearly demonstrate that NK cells arise very early during embryogenesis and that fetal NK cells are capable of both spontaneous MHC-unrestricted cytotoxicity and ADCC. Since maternal Ig can cross the placenta, NK cell-mediated ADCC may represent a significant immunological defense mechanism against viral and bacterial infections, before acquisition of T cell-mediated cellular immunity. While we have not as yet analyzed freshly isolated fetal NK cells for the capacity to produce cytokines, fetal NK clones do possess the ability to secrete IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$  (H. Spits, T. Hori, and J. Phillips, unpublished observations). Thus, it is conceivable that NK cells provide a source of cytokines that may influence development and function of the fetal immune and hematopoietic systems. Due to the difficulty in obtaining human embryonic tissue before 6 wk gestation, we were unable to determine exactly when NK cells initially become detectable in the developing embryo. It is tempting to speculate that NK cells may initially

arise from the primitive blood islands of the yolk sac during the very first wave of hematopoiesis.

Clearly the most unexpected feature of embryonic/fetal NK cells was the prominent expression of cytoplasmic CD3 $\epsilon$  and CD3 $\delta$  proteins. While CD3 $\zeta$  has been shown to coassociate with CD16 in NK cells (11, 25), adult NK cells from both mice and humans have been reported to lack expression of other CD3 subunits (6, 26). Moreover, although low levels of CD3 $\epsilon$ , but not CD3 $\delta$  or CD3 $\gamma$ , mRNA have been identified in adult human NK clones, there has been no evidence for expression of CD3 $\epsilon$  protein in these cells (26). Our present findings show that the majority of embryonic and fetal human NK cells and a small percentage of newborn cord blood NK cells express CD3 $\epsilon$ , CD3 $\delta$ , and CD3 $\zeta$  cytoplasmic antigens. We have confirmed the expression of CD3 $\epsilon$  and CD3 $\delta$  in the fetal NK clones by immunofluorescence, PCR detection of transcripts, and Western blot analysis. Unfortunately, we were unable to directly assess the expression of CD3 $\gamma$  antigen in fetal NK cells, since anti-CD3 $\gamma$  reagents that could detect cytoplasmic protein by immunofluorescent staining were unavailable. However, PCR analysis revealed the presence of CD3 $\gamma$  transcripts in some fetal NK clones, whereas CD3 $\gamma$  mRNA was essentially undetectable in other clones. Preliminary studies using an anti-CD3 $\gamma$  peptide antiserum for Western blot analysis failed to detect a mature CD3 $\gamma$  glycoprotein in lysates prepared from fetal NK clones that expressed abundant CD3 $\epsilon$  and CD3 $\delta$  cytoplasmic proteins (L. Lanier and J. Phillips, unpublished observations). However, further quantitative analysis of CD3 $\gamma$  mRNA expression and a careful study using specific anti-CD3 $\gamma$  antibodies is certainly required before it is appropriate to make any definitive conclusions.

The expression of CD3 subunits in fetal NK cells is an intriguing observation, particularly since the expression of cytoplasmic CD3 $\epsilon$  has been previously been used as an unambiguous marker to assign malignant and normal lymphocytes to the T lineage. It should be noted that all of the embryonic and fetal NK cells examined expressed CD7. Haynes et al. (33) have previously used immunohistochemical techniques to describe a population of lymphocytes in fetal liver and spleen with the phenotypes CD45<sup>+</sup>, CD7<sup>+</sup>, and cytoplasmic CD3 $\epsilon$ <sup>+</sup>. They have proposed that these cells represent pre-T cells that are committed to the T lineage and are destined for migration to the thymus. While some cells of this phenotype may be pre-T cells, our present finding would suggest that this population contains a substantial proportion of functionally competent NK cells that do not represent progenitors committed to the T cell lineage. Evidence in support of this hypothesis can be summarized as follows. (a) Most CD7<sup>+</sup>, CD45<sup>+</sup> lymphocytes in fetal liver coexpress CD56. While CD56 can be expressed on a small subset of adult peripheral blood T cells (9), mCD3 $\epsilon$ <sup>+</sup>, CD56<sup>+</sup> T cells were essentially undetectable in all fetal tissues, including thymus. Moreover, mCD3 $\epsilon$ <sup>+</sup>, CD7<sup>+</sup>, CD45<sup>+</sup> thymocytes do not express CD56 (34). (b) The population of mCD3 $\epsilon$ <sup>+</sup>, CD56<sup>+</sup> (CD7<sup>+</sup>, CD45<sup>+</sup>) fetal lymphocytes demonstrated spontaneous and IL-2-activated NK cytotoxicity and ADCC.



**Figure 11.** Two-color antigenic phenotype of fetal, newborn, and adult NK cells. Mononuclear leukocytes from a 16 wk gestation fetal liver (*A*), low buoyant density lymphocytes from newborn cord blood (*B*), and low buoyant density lymphocytes from adult peripheral blood were analyzed by two-color immunofluorescence. In *A*, there were 20% NK cells ( $mCD3\epsilon^{-}, CD56^{+}$ ) and 10% T cells ( $mCD3\epsilon^{+}, CD56^{-}$ ). These NK cells did not express CD57, however, 50% expressed low surface density CD16, and 98% expressed CD69. In *B*, 40% of the low buoyant density cord blood lymphocytes were NK cells. Newborn cord blood NK cells did not express CD57; however, they expressed low density CD69 and high surface density CD16 on the majority of cells. In *C*, 45% of the adult peripheral blood low buoyant density cells were NK cells. In this donor, 70% of the NK cells expressed CD57, 98% expressed CD16, and <10% expressed low surface density CD69.

It seems unlikely that undifferentiated progenitor cells would mediate these functions. (c) Fetal  $mCD3\epsilon^{-}, CD56^{+}$  NK cells did not express the CD34, TdT, or HLA-DR proteins; all of which have been associated with progenitor populations (35–38) and shown to be expressed on  $mCD3\epsilon^{-}, CD7^{+}$ ,

CD45<sup>+</sup> thymocytes (34). (d) None of the long-term clones established from the  $mCD3\epsilon^{-}, CD56^{+}$  fetal liver population acquired expression of TCR proteins or rearranged TCR genes. All maintained a stable NK antigenic phenotype and cytolytic function.

Collectively, these results caution against using expression of cytoplasmic CD3 proteins as definitive markers of the T cell lineage. However, these observations do support the possibility that NK and T lymphocytes arise from a common progenitor, before rearrangement of TCR genes in the T lineage. We propose that this hypothetical progenitor may reside in the fetal liver or adult bone marrow and give rise to (CD56<sup>-</sup>) cells that express cytoplasmic CD3 $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  proteins, before commitment to the NK or T lineage. As a consequence of as yet undefined signals, cells destined for the T lineage migrate to the thymus and initiate rearrangement of the TCR genes. By contrast, it is possible that progenitor cells not receiving these instructions or possibly responding to other stimuli downregulate expression of CD3 $\delta$ ,  $\gamma$ , and  $\epsilon$  and become committed to the NK lineage. If this hypothetical scheme is correct, CD3 $\gamma$  may be downregulated first during commitment to the NK lineage, followed by loss

of CD3 $\delta$  and CD3 $\epsilon$  proteins. In the majority of fetal NK cells and in a minor subset of cord blood NK cells, expression of cytoplasmic CD3 $\delta$  and CD3 $\epsilon$  proteins persists, whereas both are apparently lost in the mature, adult NK populations in blood and spleen. An alternative possibility is that the cytoplasmic CD3 $\delta$ ,  $\epsilon$ <sup>+</sup> fetal NK cells represent a distinct subpopulation of NK cells that is predominant in the fetus, but replaced by the cytoplasmic CD3 $\delta$ ,  $\epsilon$ <sup>-</sup> adult NK population after embryogenesis, comparable to the "waves" of  $\gamma/\delta$ -TCR and  $\alpha/\beta$ -TCR T cell populations that have been described during T cell development (39, 40). The functional significance of CD3 $\epsilon$  and CD3 $\delta$  cytoplasmic proteins in fetal and cord blood NK cell is unknown. These may simply represent vestigial structures that are slowly eliminated as a consequence of maturation after the hypothetical NK/T cell progenitor has committed to the NK lineage.

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