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Stochastic Acquisition of Qa1 Receptors During the Development of Fetal NK Cells In Vitro Accounts in Part But Not in Whole for the Ability of These Cells to Distinguish Between Class I-Sufficient and Class I-Deficient Targets[1]

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Fetal mouse NK cells are grossly deficient in the expression of Ly49 molecules yet show a limited ability to distinguish between wild-type and MHC class I-deficient target cells. In this paper we report that during their development in vitro from immature thymic progenitors, a proportion of C57BL/6 fetal NK cells acquires receptors for a soluble form of the nonclassical class I molecule $Qa1^b$ associated with the Qdm peptide, but not for soluble forms of the classical class I molecules K^b and D^b. The acquisition of these Qa1 receptors occurs in a stochastic manner that is strictly controlled by cytokines, and in particular is strongly inhibited by IL-4. All fetal NK clones tested, including those that lack detectable Qa1 receptors, express mRNA for CD94 and for both inhibitory and noninhibitory members of the NKG2 family. Fetal NK cells lacking receptors for Qa1 (and also for classical class I molecules) cannot distinguish between wild-type and class I-deficient blasts but, surprisingly, distinguish efficiently between certain wild-type and class I-deficient tumor cells. A variant line that lacks several members of the NKG2 family kills both types of tumor cell equally well, suggesting the existence of NKG2-containing inhibitory receptors that recognize as yet undefined nonclassical class I molecules of restricted distribution. *The Journal of Immunology,* **1999, 163: 3176–3184.**

It has become clear in recent years that the recognition mechanisms used by NK cells are much more complex than previously imagined (1). The ability of NK cells to destroy certain malignant, infected, and foreign cells appears to be regulated by a balance between activating and inhibitory signals (2). The latter are delivered by a series of receptors that recognize self MHC class I molecules. In this way, target cells that express a normal complement of class I molecules do not trigger effector responses in NK cells, leading to the notion, originally referred to as the "missing self hypothesis," that a major function of NK cells is to eliminate cells that are defective in expression of class I molecules (3, 4).

In humans, two sets of inhibitory receptor have been identified. One set belongs to the Ig superfamily and recognizes determinants found on classical class I molecules (5). The second set belongs to the C-type lectin family and comprises an invariant CD94 chain covalently linked to a member of the NKG2 family (6). CD94/NKG2 receptors were initially thought to recognize determinants shared by many classical class I molecules, but it has now been established that they recognize the nonclassical HLA-E molecule whose expression at the cell surface is largely dependent on its association with a peptide that is liberated by intracellular proteolytic digestion of the leader sequence of certain classical class I molecules (7–9).

Until recently the only class I receptors known to be expressed on murine NK cells were those belonging to the Ly49 family (10, 11). Those members of the Ly49 family that have been studied in detail have been found to recognize particular classical class I molecules and to be expressed on overlapping subpopulations of cells, raising important questions as to how a complex and diverse repertoire of functional self-tolerant NK cells is created during ontogeny. It is currently believed that during a critical stage in their development, NK cells activate Ly49 genes at random from the entire set of genes available on both homologous chromosomes. The resulting immature NK cell repertoire is then acted upon by a selection process that results in the survival only of those NK cells that express at least one inhibitory receptor for self class I molecules (12–14).

Understanding the nature of these events would be greatly facilitated if it were possible to study the development of NK cells in vitro from individual precursors. We have recently developed a system in which immature progenitor cells from day 14 fetal thymus (15) or liver (16) can develop into lines and clones of NK cells in vitro. The NK cells that arise in this system are phenotypically

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and functionally similar to adult splenic NK cells with two striking exceptions, namely that they are severely deficient in the expression of Ly49 molecules and show only limited ability to discriminate between class I-sufficient and class I-deficient targets (17). Thus, most fetal NK lines lyse syngeneic blast cells almost as well as blasts prepared from class I-deficient mice. Curiously though, all lines and clones of fetal NK cells that we examined gave strong lysis of a β_2-microglobulin (β_2m)[3]-deficient mutant of EL4 tumor cells and of a TAP2-deficient mutant of RBL5 tumor cells, but little or no lysis of the corresponding wild-type cells. These results strongly suggest that fetal NK cells express inhibitory class I receptors distinct from those previously characterized. One possibility would be that they express novel member(s) of the Ly49 family. In support of this we found that, although fetal NK cell lines generally lacked detectable levels of most Ly49 molecules, both at the cell surface and at the RNA level, they expressed high levels of mRNA for Ly49E (17). Alternatively, they may express inhibitory receptors homologous to those found in man, or ones of a novel nature.

The recent demonstration that adult mouse NK cells express inhibitory CD94/NKG2 receptors that recognize Qa1 (18, 19), the mouse homologue of HLA-E, led us to investigate the expression of receptors for Qa1 on fetal NK cells. Our studies reveal that, during their development in vitro, a proportion of C57BL/6 fetal NK cells do indeed acquire receptors for a soluble form of $Qa1^b$ bearing the Qdm peptide (hereinafter referred to as Qa1R), but not for soluble forms of syngeneic classical class I molecules. The acquisition of these Qa1R is under strict cytokine control, occurs in a stochastic manner, and correlates with the limited ability of fetal NK cells to distinguish between wild-type and class I-deficient blasts. However, these Qa1R do not account for the striking ability of fetal NK cells to discriminate between class I-sufficient and class I-deficient forms of certain tumor cells, suggesting the existence on NK cells of novel inhibitory receptors for structures of an unknown nature involving nonclassical class I molecules.

Materials and Methods

Culture medium and reagents

Culture reagents were purchased from Life Technologies (Paisley, U.K.) and Sigma (Poole, U.K.). Human rIL-2 was obtained from Cetus (Emeryville, CA). Mouse rIL-4 was obtained as the supernatant of X6310 cells transfected with the mouse IL-4 gene (20), kindly provided by Dr. F. Melchers (Basel Institute for Immunology, Basel, Switzerland).

Adult and fetal NK cells

Adult NK cells were prepared as described previously (17). Fetal NK cells were generated as described in detail elsewhere (15, 21). Briefly, thymocytes were prepared from day 14 embryos of timed-mated C57BL/6 mice (day of vaginal plug = day 0), and, unless stated otherwise, were cultured for 1–3 days in medium containing 10 U/ml IL-4 and 10 ng/ml PMA, then transferred to medium containing 10^4 U/ml IL-2.

CTL clones

The Qdm-dependent Qa1-specific clone e1d (22) and the H-Y-specific clone CTL10 ((23); kindly provided by Dr. D. Roopenian) were maintained by periodic stimulation with irradiated spleen cells from male C57BL/6 mice in medium containing 100 U/ml IL-2. After 1 wk of growth, cells were transferred to medium containing 5 U/ml IL-2. Cells were used in experiments while growing in 100 U/ml IL-2.

Immunofluorescence and flow cytometry

Aliquots of 1–3 $\times$ 10^5 cells were stained with combinations of FITC PK136 anti-NK1.1, PE 2C11 anti-CD3ϵ, and PE or Red670 tetrameric class I molecules. Abs were obtained from PharMingen (San Diego, CA) or Cambridge Bioscience (Cambridge, U.K.). Soluble tetramers of $Qa1^b$ (refolded with the Qdm peptide, AMAPRTLLL) and K^b (construct kindly provided by Dr. J. D. Altman, Emory University, Atlanta, GA; refolded with the OVA peptide, SIINFEKL) associated with Red670 streptavidin (Life Technologies) and of D^b (refolded with the H-Y peptide, WMHHMLDI) associated with PE-streptavidin were generated as described elsewhere ((18); D. Sourdive et al. in preparation). Staining was analyzed on a FACScan (Becton Dickinson, San Jose, CA), using forward and side scatter to gate on single viable cells. Compensation for spectral overlap of dyes was set by running mixtures of unstained cells and cells stained with each of the fluorochromes singly. $Qa1R^+$ and $Qa1R^-$ cells were purified by sorting Qa1-tetramer stained fetal NK cells on a FACS Vantage (Becton Dickinson).

RT-PCR

Total cytosolic RNA was extracted from $\sim 1 \times 10^6$ cells using Trizol (Life Technologies). Ten micrograms of DNase-treated RNA was reverse transcribed using an oligo$(dT)_{12-18}$ primer and a Superscript preamplification kit (Life Technologies). PCR amplification was performed on aliquots of cDNA using a hot start after an initial denaturation at 95°C, followed by 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 50 s, with a final extension time of 7 min. The Ly49E primers have been described previously (17). The forward and reverse primers for CD94 and NKG2A, NKG2C, and NKG2D were based on sequences obtained by Vance et al. (24) and Lohwasser et al. (25) and were as follows: CD94f, TCTAGGATCACTCGGTGGAG; CD94r, AACCGTGGACCTTCCTTGTC; NKG2Af, CTATGCAGAACTGAAGGTG; NKG2Cf, CTGCTTGGAACTGAACAGG; NKG2A/Cr, CCAAGATTTTCTCTCCATAC; NKG2Df, GAGCAAATGCCATAATTACG; and NKG2Dr, CATAGACAGCACAGGATCC. Products were analyzed on 3% NuSieve GTG agarose (FMC BioProducts, Chicago, IL) containing ethidium bromide.

Cytotoxicity assays

These assays were performed in a standard manner by incubating serial dilutions of effector cells with 5000 ^{51}Cr-labeled target cells for 4 h in V-bottom microtest plates. The C57 lymphoma lines EL4 and RMA, together with the β_2m-deficient mutant of EL4, C4.4 (26), and the TAP-deficient mutant of RMA, RMA/S (3), were kindly provided by Drs. R. Glas and K. Kärre (Karolinska Institute, Stockholm, Sweden). RMA/S cells transfected with mouse CD1 cDNA (27), and the 1B1 anti-CD1 mAb (28) were kindly provided by Dr. M. Kronenberg (Los Angeles, CA). The F12 cell line transfected with a hybrid $Qa1^b/D^b$ construct has been described previously (22). During extended cultivation, this cloned transfected L cell line spontaneously generated a $Qa1^b/D^b-$ve revertant subpopulation. Expressing and nonexpressing cells were separated on a FACS Vantage following staining with HB27 anti-D^b mAb (American Type Culture Collection, Manassas, VA), and were returned to culture to give the $F12^+$ and $F12^-$ lines. T2 cells transfected with the $Qa1^b$ gene (29), and control T2 cells were kindly provided by Dr. M. Soloski (Johns Hopkins, Baltimore, MD). Before cytotoxicity assays, these cells were incubated overnight at 26°C in AIMV medium containing 30 μM Qdm peptide. Chromium labeling and cytotoxicity assays were also performed in the presence of 30 μM peptide. Blast cells were prepared from frozen CD8-depleted spleen cells of C57BL/6 mice and mice homozygous for the β_2m knockout mutation on a C57 background, kindly provided by Prof. E. Jenkinson (Birmingham, U.K.). Thawed spleen cells were cultured for 2–3 days in αMEM containing 20 mM HEPES, 5×10^{-5} M 2-ME, 10% FBS, 2 μg/ml Con A, 50 μg/ml gentamicin, and 100 U/ml IL-2.

Results

Fetal NK cells from C57BL/6 mice can bind to a soluble form of the nonclassical class I molecule, Qa1, but not to soluble forms of the classical class I molecules K^b and D^b

The left hand part of Fig. 1 shows typical results obtained when polyclonal fetal and adult NK cell preparations derived from C57BL/6 mice were stained with soluble tetrameric forms of K^b, D^b, and $Qa1^b$. As reported previously (18, 19), ~50% of adult NK cells bore receptors that could be detected with Qa1-Qdm tetramers. In most polyclonal fetal NK cell lines that had been cultured for more than 7 days, ~35% of cells bore Qa1R of this type, the level of staining being similar to that on adult NK cells. By contrast, although ~40% of adult NK cells showed clear staining with a K^b tetrameric construct, few if any fetal NK cells stained. Neither

[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; Qa1R, receptors for soluble $Qa1^b$-Qdm complexes.

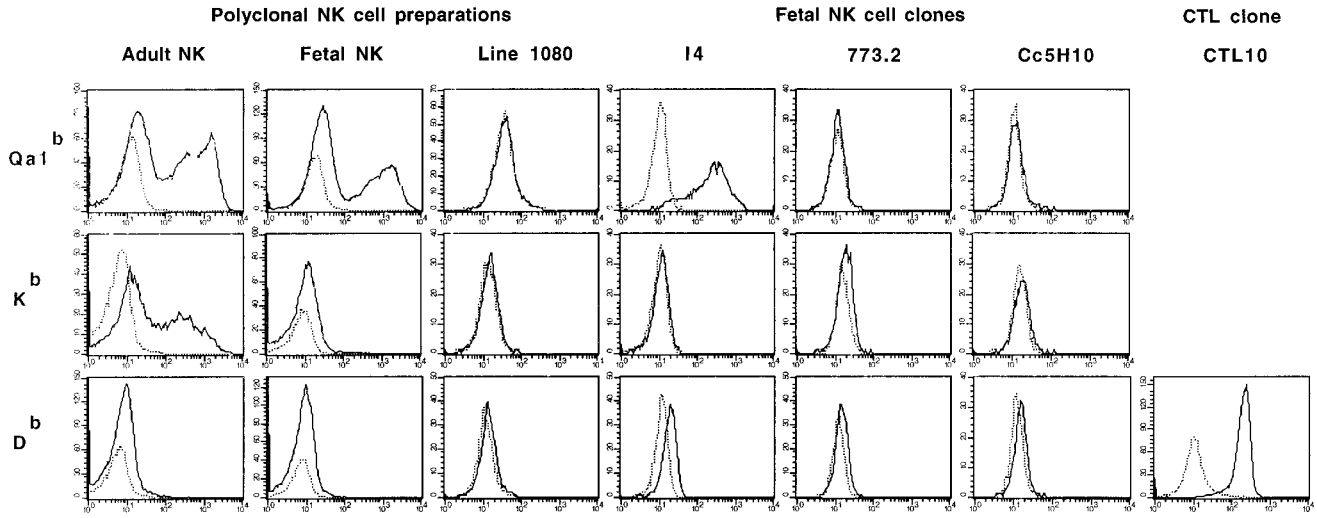

FIGURE 1. Fetal NK cells express receptors for Qa1 but appear to be devoid of receptors for K^b and D^b. Polyclonal adult and fetal NK cells that had been cultured for 10 days in IL-2, the long-term fetal NK line 1080, fetal NK clones I4, 773.2, and Cc5H10, and the H-Y/D^b-specific T cell clone CTL10 were stained with soluble tetrameric preparations of $Qa1^b$, K^b, and D^b (solid lines) or medium alone (dotted lines).

adult nor fetal NK cells showed any detectable staining with a D^b tetrameric construct that stained the cognate T cell clone CTL10. An exception to the rule that fetal NK cell lines contained $Qa1R^+$ cells was the long-term fetal NK cell line 1080.

Qa1R-expressing cells arise in a time and cytokine-dependent manner during the development of NK cells from fetal thymic progenitors

The fetal NK cell lines studied here were derived by culturing day 14 fetal thymocytes in IL-4 + PMA then transferring them to high-dose IL-2. The changes that occur in the expression of NK1.1 and Qa1R during the early stages of this process are illustrated in Fig. 2 and summarized in Table I. Before culture, only about 0.7% of fetal thymocytes had an NK phenotype ($CD3^-$ $NK1.1^+$), but interestingly ~20% of these cells expressed Qa1R. Following culture for 2 days in IL-4 + PMA, the frequency of both $CD3^-$ $NK1.1^+$ cells and the proportion of these expressing Qa1R did not change. Thus, only about 0.1% of the cells present at this stage were $CD3^-$ $NK1.1^+$ $Qa1R^+$ cells. However, within 24 h of transfer to medium containing IL-2, 23% of cells had acquired NK1.1 and 12% of these bore Qa1R. Thus, about 2.7% of the cells in culture were now $CD3^-$ $NK1.1^+$ $Qa1R^+$ cells. Because there is no noticeable cell death when cells are transferred to IL-2, and 50–70% of input cells proliferate under these conditions (15), the data strongly suggest that the dramatic increase in the proportion of both $CD3^-$ $NK1.1^+$ cells and $CD3^-$ $NK1.1^+$ $Qa1R^+$ cells that occurs at this stage is due to the de novo expression of NK1.1 and Qa1R on progenitor cells rather than the selective growth or survival of preexisting cells of this type. This conclusion was directly confirmed by single cell experiments (see below).

Over the next few days the proportion of $NK1.1^+$ and $NK1.1^+$ $Qa1R^+$ cells continued to increase, but more slowly (Table I). Throughout the culture period, the expression of Qa1R was largely restricted to cells that expressed NK1.1. The addition of PMA accelerated the rate at which cells acquired expression of NK1.1 and Qa1R. By contrast, the addition of IL-4 had no effect on the acquisition of NK1.1 but completely blocked the acquisition of Qa1R. Even more remarkably, when cells that had been grown in IL-2-containing secondary cultures for 3 days were transferred to medium containing IL-4, the expression of Qa1R was virtually extinguished within 3 days. Kinetic analysis (data not shown) re-

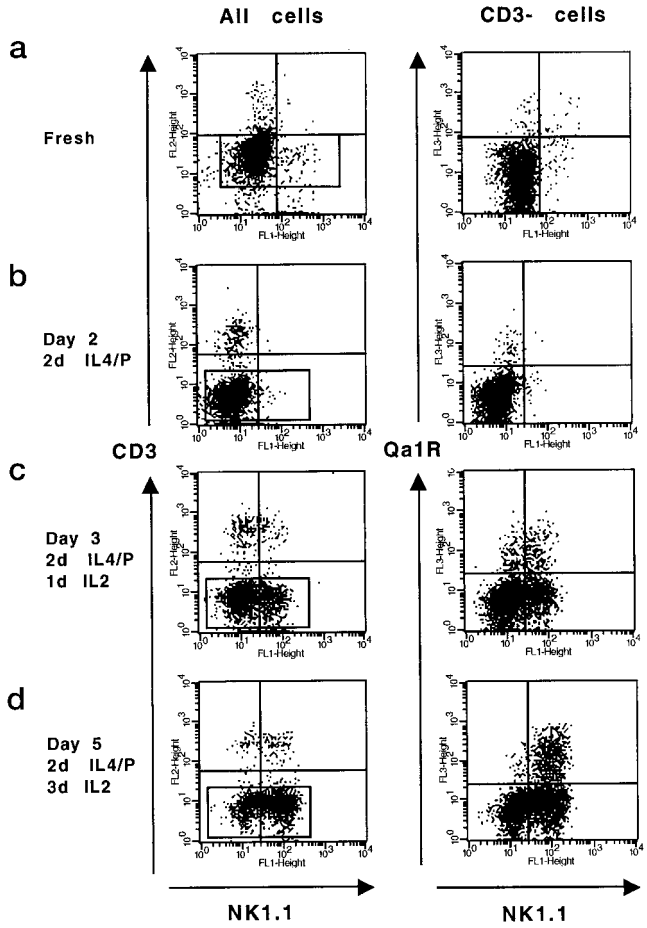

FIGURE 2. The development of NK cells expressing Qa1R occurs rapidly when IL-4/PMA-primed fetal thymocytes are exposed to IL-2. Day 14 fetal thymocytes were triple stained with FITC anti-NK1.1, PE anti-CD3ε, and Red670 Qa1-tetramer after no culture (*a*), 2 days of culture in IL-4 + PMA (*b*), a further 1 day of culture in IL-2 (*c*), or a further 3 days of culture in IL-2 (*d*). The plots on the *right-hand side* show the NK1.1 and Qa1R expression of $CD3^-$ cells defined by the rectangular regions shown in the CD3 vs NK1.1 plots on the *left hand side*. For quantitative analysis of the data, see Table I.

Table I. *Conditions that control the development of NK1.1$^+$ and Qa1R$^+$ cells from fetal thymus progenitors in vitro*

First Culture	Second Culture	Third Culture	% of All Cells That Are CD3$^-$ NK1.1$^-$	% of CD3$^-$ NK1.1$^-$ Cells That Are Qa1R$^+$	% of All Cells That Are CD3$^-$ NK1.1$^+$	% of CD3$^-$ NK1.1$^+$ Cells That Are Qa1R$^+$	% of All Cells That Are CD3$^-$ NK1.1$^+$ Qa1R$^+$
Fresh			95.5	0.6	0.7	18.2	0.1
2 days in IL-4 + PMA	0	0	93.2	2.1	0.6	16.1	0.1
2 days in IL-4 + PMA	1 day in IL-2	0	67.7	3.0	23.1	11.6	2.7
2 days in IL-4 + PMA	3 days in IL-2	0	37.2	2.6	55.0	22.4	12.3
2 days in IL-4 + PMA	3 days in IL-2 + PMA	0	8.6	11.2	85.2	37.8	32.2
2 days in IL-4 + PMA	3 days in IL-2 + IL-4	0	35.7	0.2	53.5	0.6	0.3
2 days in IL-4 + PMA	3 days in IL-2	3 days in IL-2	7.1	4.6	88.3	37.2	32.8
2 days in IL-4 + PMA	3 days in IL-2	3 days in IL-2 + IL-4	2.5	0.0	94.0	3.1	2.9
2 days in IL-4 + PMA	3 days in IL-2 + IL-4	3 days in IL-2 + IL-4	7.3	0.1	87.7	0.3	0.3
2 days in IL-4 + PMA	3 days in IL-2 + IL-4	3 days in IL-2	7.4	0.2	88.7	1.1	1.0

vealed that, following transfer to medium containing IL-4, the proportion of Qa1R$^+$ cells declined 2- to 3-fold each day. Cells that had been grown in secondary cultures containing IL-2 + IL-4 for 3 days did not acquire Qa1R upon subsequent transfer to medium lacking IL-4 (at least within 3 days). Thus, IL-4 exerts rapid, dramatic, and largely irreversible effects on the expression of Qa1R on developing NK cells.

Single cell experiments demonstrate that developing NK cells acquire Qa1R in a stochastic manner

The finding that Qa1R are only expressed on a subpopulation of NK cells that develop in vitro has two possible explanations. The first is that Qa1R$^+$ and Qa1R$^-$ NK cells arise from separate committed precursors that, respectively, switch on or do not switch on Qa1R expression during development. The second is that Qa1R$^+$ and Qa1R$^-$ NK cells arise from a common precursor whose descendants randomly switch on Qa1R during a critical stage of development. To determine which of these possibilities was correct, IL-4/PMA-primed fetal thymocytes that consist of >99% NK1.1$^-$ Qa1R$^-$ cells were cloned in IL-2 at an average of 0.1 cell/well.

Poisson analysis indicated that >99% of the developing NK cell clones arose from single progenitors. Because ~30% of the cells that were plated gave rise to clones, the vast majority of clones must therefore have arisen from NK1.1$^-$ Qa1R$^-$ progenitors. Twelve of 12 clones tested showed uniform expression of NK1.1 on all cells. Of 34 clones examined for expression of Qa1R (which included the 12 clones examined for NK1.1), 31 expressed Qa1R. Importantly, every one of these 31 clones displayed mosaic expression of Qa1R. The results for 10 randomly chosen clones are shown in Fig. 3. The percentage of Qa1R$^+$ cells within these clones ranged from 3% (J1.4) to >90% (J1.1). When heterogeneous clones were restained after a further 2 wk of culture, the proportions of Qa1R$^+$ and Qa1R$^-$ cells were very similar to what was originally found (data not shown). By contrast, 4/6 long-established fetal NK clones that were examined contained no detectable Qa1R$^+$ cells, suggesting that Qa1R may be lost during long-term culture. The results for two of these, 773.2 and Cc5H10, are shown in Fig. 1, together with the results for one long-term clone, I4, that contained >95% Qa1R$^+$ cells.

Fetal mouse NK clones express mRNA for CD94 and at least three members of the NKG2 family

Compelling evidence has been provided that tetrameric molecules formed from soluble forms of Qa1, or its human homologue HLA-E, bind to CD94/NKG2 heterodimeric receptors on NK cells (7, 19). To determine whether fetal NK cell lines and clones express CD94 and NKG2, RT-PCR was performed on total cytosolic RNA extracted from appropriate cells. Key results are shown in Fig. 4. Of 13 polyclonal and monoclonal fetal NK lines examined, all expressed mRNA for CD94. Polyclonal adult and fetal NK cell lines also expressed mRNA for NKG2A, NKG2C, and NKG2D. The single exception was the polyclonal line 1080, which lacked detectable mRNA for each of these NKG2 species. In addition to the major bands, whose identity was confirmed by sequencing, PCR amplification of NKG2 mRNA frequently generated additional weak bands. In particular, the NKG2A primers invariably gave rise to a subsidiary band ~100 bp smaller than the correct NKG2A amplimer. This band was close to the size expected for NKG2B, a putative alternately spliced form of NKG2A. However, sequencing revealed it to be derived from an NKG2A-like transcript that lacked nt 188–289 of the published NKG2A sequence. This deletion creates a potential in-frame translation product lacking the transmembrane domain.

Remarkably and importantly, all 10 clones examined (the results for 5 of which are shown in Fig. 4) expressed mRNA for both putative inhibitory (NKG2A and NKG2D) and noninhibitory (NKG2C) NKG2 molecules. Expression, and indeed, as far as could be judged, the level of expression, bore no relationship to the level of expression of surface Qa1R. In particular, it was quite clear that clones that contained few (J1.4) or no (773.2 and Cc5H10) detectable Qa1R$^+$ cells contained mRNA for CD94 and for NKG2A, NKG2C, and NKG2D, although in the case of clone

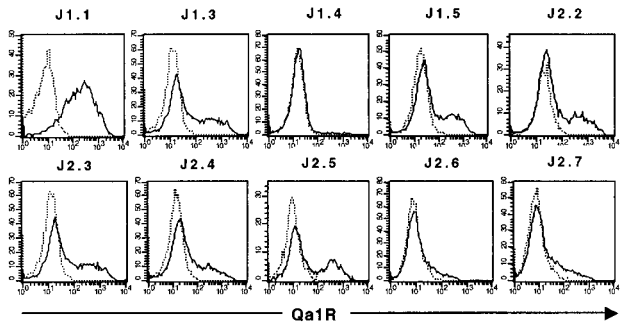

FIGURE 3. Qa1R expression on a set of 10 randomly selected fetal NK clones. Cells were stained with Qa1 tetramer (solid line) or medium alone (dotted line).

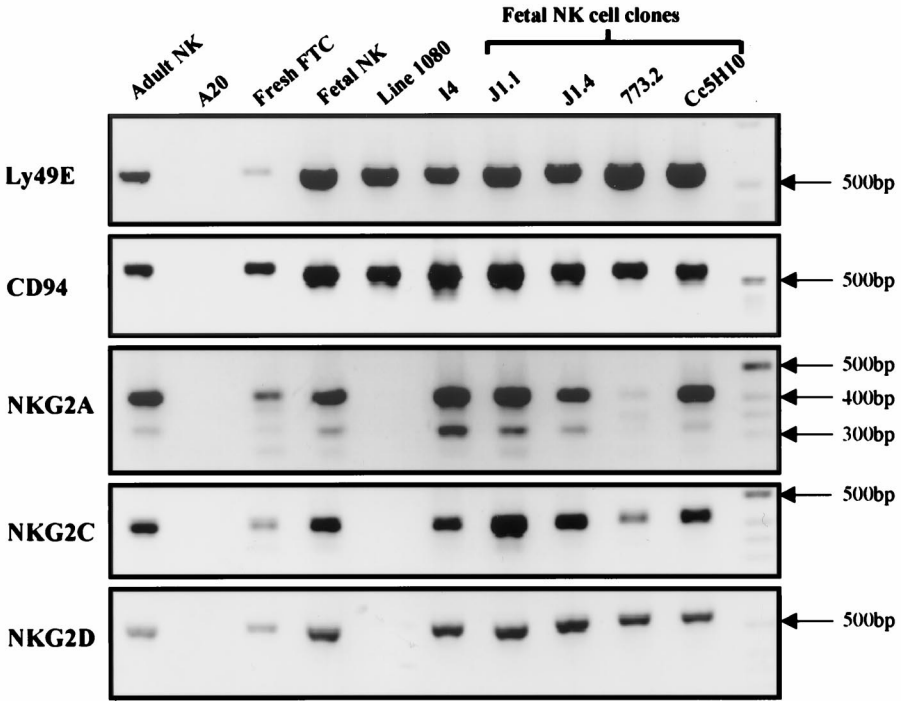

FIGURE 4. RT-PCR analysis of Ly49E, CD94, and NKG2 expression in adult and fetal NK cells. Total cytosolic RNA was prepared from ~1 million cells and reverse transcribed using an oligo(dT) primer. The resulting cDNA was tested in PCR with specific primers for Ly49E, CD94, NKG2A, NKG2C, and NKG2D. The cells used were adult NK cells, the A20 mouse B cell lymphoma, fresh day 14 fetal thymocytes (FTC), polyclonal fetal NK cells, fetal NK line 1080, the predominantly Qa1R$^+$ clones I4 and J1.1, the predominantly Qa1R$^-$ clone J1.4, and clones 773.2 and Cc5H10 that contained no detectable Qa1R$^+$ cells.

773.2 the NKG2A and NKG2C signals were low. Each of the polyclonal and monoclonal NK cell lines was also examined for expression of mRNA encoding Ly49E, the only member of the Ly49 family known to be expressed in these cells. All were strongly positive, including the 1080 line that lacked expression of NKG2 molecules. RT-PCR analysis was also performed on fresh day 14 fetal thymocytes. Despite the very low abundance of fetal thymocytes with NK characteristics, unexpectedly strong signals were found for CD94 and NKG2A, with weaker but clear signals for NKG2C and NKG2D, and for Ly49E (Fig. 4).

Cytolytic activity of Qa1R$^+$ and Qa1R$^-$ fetal NK cells

To determine the functionality of the Qa1R expressed on fetal NK cells, the cytolytic specificity of the cells bearing them was examined. Representative results of a large series of experiments are shown in Fig. 5. As reported previously, polyclonal fetal NK cells, in contrast to polyclonal adult splenic NK cells, distinguished only weakly if at all between wild-type and β_2m-deficient blast cells. By contrast, purified Qa1R$^+$, but not Qa1R$^-$, fetal NK cells preferentially lysed β_2m-deficient blasts. The most likely interpretation of this result is that many of the fetal NK cells that bear Qa1R express inhibitory forms of these receptors that prevent the cells that bear them from lysing Qa1$^+$ blasts. As shown by using the Qa1-specific T cell clone, e1d, most if not all of the cells in the wild-type blast cell population did indeed express Qa1, whereas β_2m-deficient blast cells lacked Qa1 molecules that could be recognized by e1d. The fact that wild-type blast cells were still killed to a significant extent by purified Qa1R$^+$ fetal NK cells could be because the Qa1R$^+$ cells were contaminated with some Qa1R$^-$ cells, the Qa1R expressed by some Qa1R$^+$ cells are noninhibitory, or the inhibitory signals delivered via Qa1R are not completely effective. Clearer results were obtained using cloned fetal NK cells lines. The predominantly Qa1R$^+$ clone J1.1 discriminated very clearly between wild-type and β_2m-deficient blasts, whereas the clone J1.4, and also the polyclonal line 1080, that were largely or completely devoid of Qa1R$^+$ cells, killed both wild-type and β_2m-deficient blasts equally well. Strikingly different results were obtained when each of these various effector cells was tested on wild-type EL4 cells and the β_2m-deficient variant of EL4, C4.4. Not only did adult NK cells and Qa1R$^+$ fetal NK cells preferentially lyse β_2m-deficient EL4 cells, but so too did Qa1R$^-$ polyclonal fetal NK cells, the Qa1R$^-$ clone J1.4, and all other fetal Qa1R$^-$ clones tested. By contrast, the 1080 line that lacked not only Qa1R$^+$ cells but also NKG2A, NKG2C, and NKG2D, killed wild-type EL4 cells more efficiently than β_2m-deficient EL4 cells.

That the preferential lysis of wild-type compared with β_2m-deficient blast cells by Qa1R$^+$ fetal NK cells was due to the recognition of Qa1 molecules expressed on these targets was supported by the results obtained with Qa1-transfected cell lines (Fig. 6). Thus, the Qa1-transfected L cell line F12$^+$ was resistant to lysis by Qa1R$^+$ clones such as I4 and J1.1, compared with a revertant Qa1$^-$ line derived from F12. That the structures on target cells that inhibited lysis by Qa1R$^+$ fetal NK cells were most likely Qa1 molecules bearing the dominant Qdm peptide was strongly suggested by the finding that the human T2 line transfected with Qa1 became resistant to both purified polyclonal Qa1R$^+$ fetal NK cells and to Qa1R$^+$ NK clones, but not to Qa1R$^-$ fetal NK cells, when incubated with Qdm peptide.

The finding that fetal NK cells that apparently lack receptors for both classical class I molecules and for Qa1-Qdm can distinguish efficiently between wild-type and β_2m-deficient EL4 cells suggests that they may bear inhibitory receptors for a structure involving a nonclassical class I molecule, distinct from Qa1-Qdm, that is expressed by EL4 cells but not by normal blast cells. It has recently been reported that the nonclassical class I molecule CD1 can inhibit killing by adherent LAK cells prepared from adult mice (30). However, not only can Qa1R$^-$ fetal NK cells distinguish between wild-type and β_2m-deficient EL4 cells, they can also distinguish

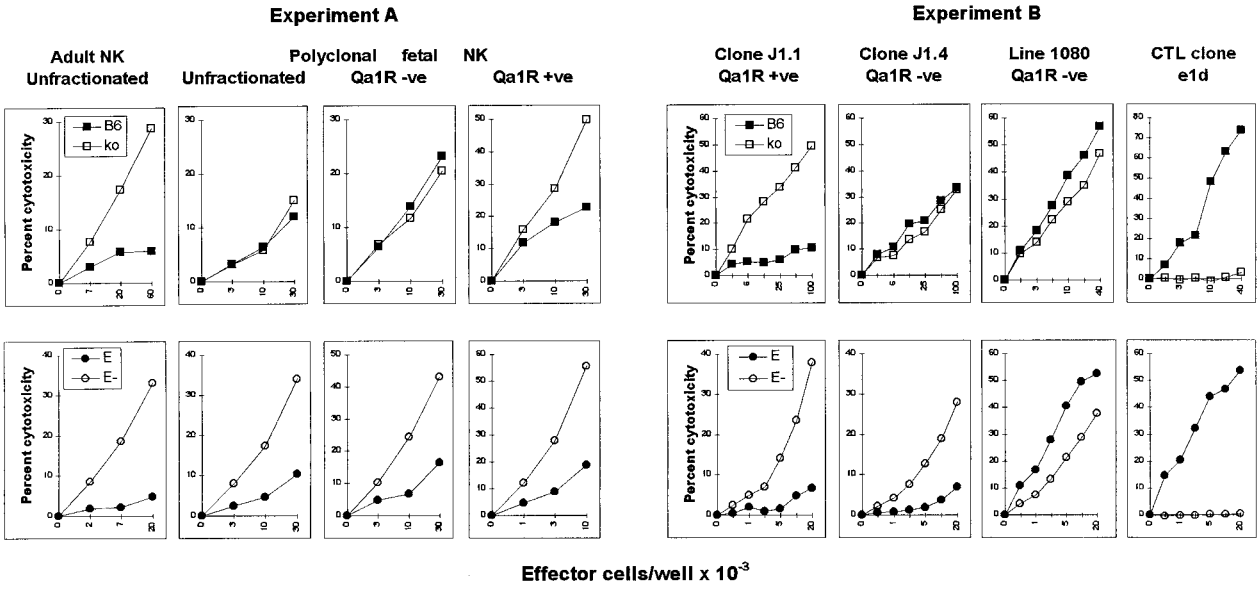

FIGURE 5. Qa1R$^-$ fetal NK cells can distinguish between wild-type and β_2m-deficient EL4 cells, but not between wild-type and β_2m-deficient blasts. Various preparations of adult and fetal NK cells, and the Qa1-specific CTL clone e1d, were tested for cytotoxicity on wild-type C57BL/6 (B6) or β_2m-deficient (ko) blast cells (*upper graphs*), and on wild-type (E) or the β_2m-deficient variant (E-) of EL4 cells, C4.4 (*lower graphs*). In experiment A the effector cells used were cultured adult NK cells together with polyclonal fetal NK cells that were either unfractionated or had been purified by cell sorting. The Qa1R −ve population was 97% pure, and the Qa1R +ve population was ~90% pure. In experiment B, the effector cells used were the predominantly Qa1R +ve clone J1.1, the predominantly Qa1R −ve clone J1.4, the Qa1R −ve line 1080, and the Qa1/Qdm-specific T cell clone e1d.

between wild-type and TAP-deficient RMA cells even though both target cells express similar levels of CD1 (Fig. 7). Furthermore, CD1-transfected RMA/S cells that vastly over-express CD1 show little or no resistance to fetal NK cells (Fig. 7). Line 1080, which fails to distinguish between wild-type and β_2m-deficient EL4 cells, is also clearly unable to distinguish between wild-type and TAP-deficient RMA cells.

Discussion

One of the principal findings reported in this study is that, during their differentiation in vitro from fetal thymic progenitors, developing NK cells acquire Qa1R in a stochastic manner. Thus, when individual IL-4/PMA-primed thymocytes, of which <1% express NK1.1 or Qa1, were seeded into wells containing IL-2, ~30%

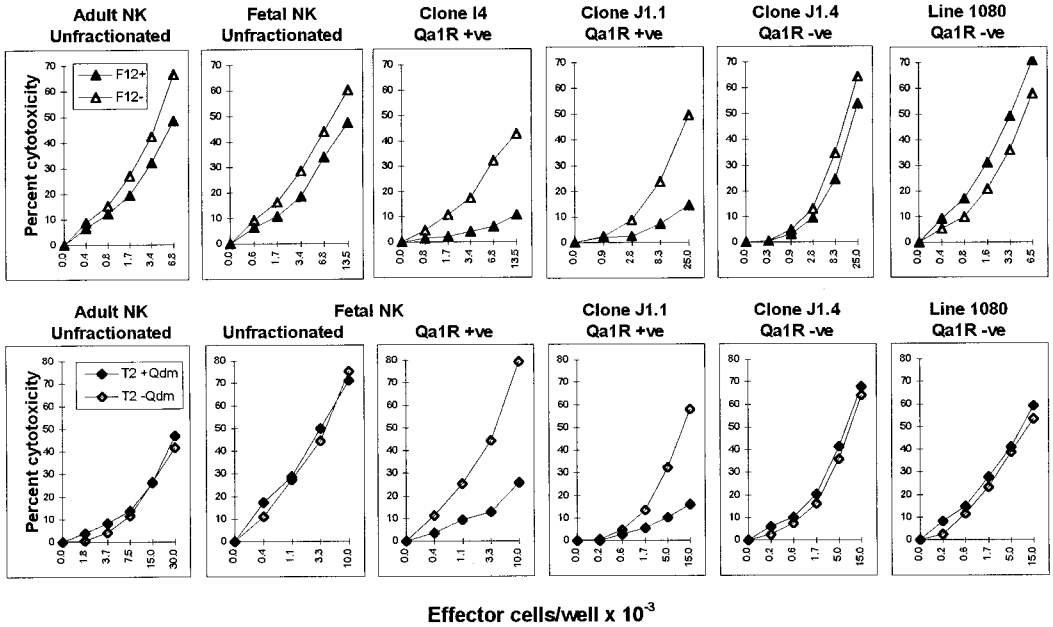

FIGURE 6. Qa1-expressing target cells are protected from lysis by Qa1R$^+$ but not Qa1R$^-$ fetal NK cells. Various preparations of adult and fetal NK cells were tested for cytotoxicity on L cells expressing a hybrid Qa1/D^b molecule (F12$^+$), a revertant line (F12$^-$) of these cells that had lost expression of both D^b and Qa1 (as determined by testing with Qa1-specific T cells clones), Qa1-transfected T2 cells that had been incubated overnight at 26°C in medium alone (T2 −Qdm), or the same cells that had been incubated in medium containing 30 μM Qdm peptide (T2 +Qdm). The effector cells used were as described in the legend to Fig. 5.

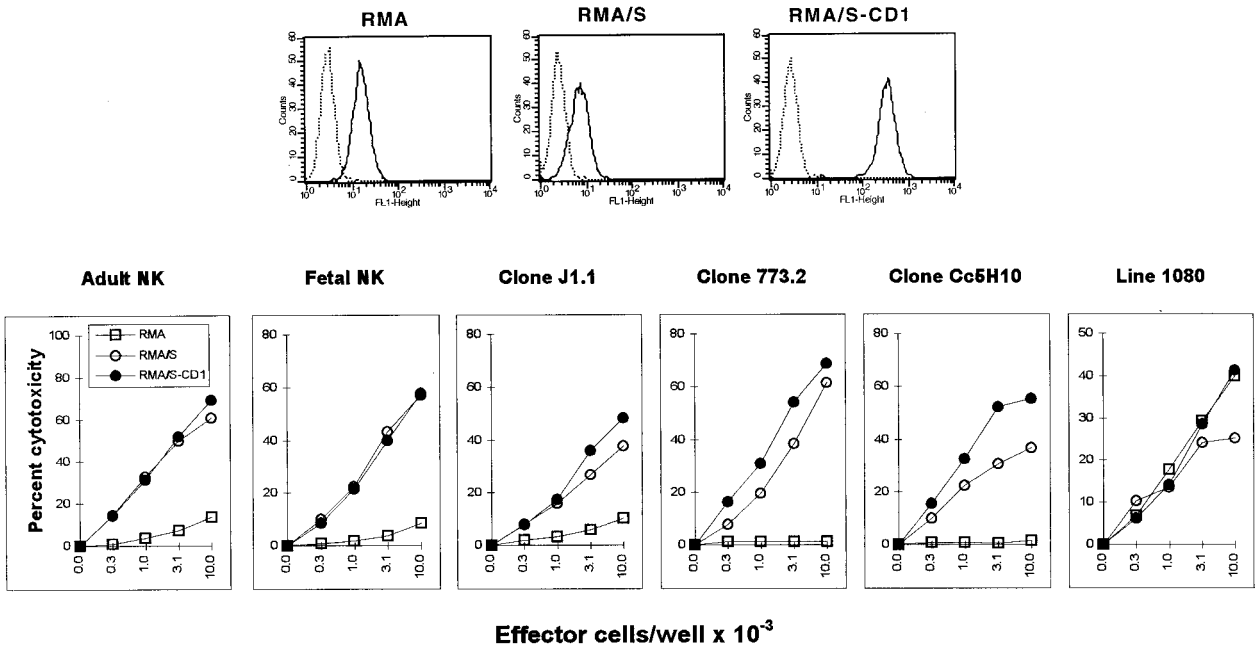

FIGURE 7. CD1 fails to protect TAP-deficient tumor cells from lysis by fetal NK cells. *Upper graphs,* The expression of CD1 on RMA, RMA/S, and RMA/S cells transfected with CD1. *Lower graphs,* Lysis of these cells by polyclonal adult and fetal NK cells, the predominantly Qa1R$^+$ clone J1.1, the Qa1R$^-$ clones 773.2 and Cc5H10, and the NKG2-deficient line 1080.

developed into clones that displayed uniform expression of NK1.1 but, in the vast majority of cases, mosaic expression of Qa1. It seems that the probability of cells switching on expression of Qa1R is highest in the first few days after transfer to IL-2, and declines substantially thereafter. Thus, within 1 day of transfer to IL-2, the proportion of NK cells that expresses Qa1R rises almost 30-fold from about 0.1% to 2.7%, but over the next 2 days it increases only 5-fold to about 12%, and over the following 3 days by less that 3-fold to 33%, after which there is no further increase no matter how long the cells are grown. Furthermore, if after 8 days of culture in IL-2, Qa1R$^-$ cells were purified to >99% purity by cell sorting and returned to culture, only about 2% acquired Qa1R even after 7 days of further culture (data not shown).

It therefore seems likely that the characteristic mosaic Qa1R expression pattern of individual clones is established early in clonal development. If we assume that the acquisition of Qa1R is largely irreversible, then a clone that contains ~50% Qa1R$^+$ cells most likely arose because one of the two daughter cells of the first IL-2-driven division acquired Qa1R. Similarly, a clone that contains about 25% Qa1R most likely arose because one of the four grand-daughter cells switched on Qa1R. The finding that about 12% of NK1.1$^+$ cells acquire Qa1R in the first 24 h (approximately two cell divisions) indicates that the probability of NK cells switching on Qa1R is initially about 0.1/division. If this model is correct it suggests that NK clones that are uniformly Qa1R positive would be rare, as they would only be likely to arise if both daughter cells of the first division acquired Qa1R. This prediction is supported by the observation that <5% of the clones examined appear even close to being uniformly Qa1R$^+$, the great majority having <50% Qa1R$^+$ cells. Coupled with a declining switching rate, perhaps associated with a declining rate of cell division, this model readily explains why no more than ~35% of the cells in fetal thymus-derived NK cell populations ever express Qa1R.

The issue of whether Qa1R expression can be switched off as well as on is an important one. The fact that clones composed predominantly of Qa1R$^+$ cells remain predominantly Qa1R$^+$ for many weeks suggests that if cells can switch off Qa1R they do so at a rate that is slow compared with the on rate in early cultures. Indeed, the observation that fetal NK clones show stable but distinctive patterns of Qa1R expression supports the deterministic model described above, and argues strongly against an alternative model in which the expression of Qa1R on ~35% of polyclonal fetal NK cells is due to a dynamic equilibrium being reached between on and off rates. Whatever mechanisms are responsible for the stochastic expression of Qa1R, it is possible that they influence the expression of other molecules. In particular, several members of the CD45, Ly6, and NKRP families have also been found to be expressed in a mosaic manner on fetal NK clones (31). The rate of switching appeared to be different for different molecules, being particularly high for Ly6C and the NKRP molecules recognized by mAb 10A7. We have also found occasional expression of Ly49 molecules on fetal NK clones, and in every case this has been of a mosaic nature (Ref. 31 and our unpublished observations).

The mosaic expression of Qa1R within NK cell clones implies that the individual cells that constitute a clone vary in their expression of one or more of the components required for the synthesis or expression of Qa1R. All available evidence indicates that CD94/NKG2 heterodimeric complexes are the principal if not sole receptors for Qa1 and its human homologue HLA-E (7–9, 19). The finding that some established NK clones lack any detectable surface Qa1R yet contain mRNA for both CD94 and NKG2 molecules indicates that Qa1R expression may be controlled, at least in part, at the posttranscriptional level. This contrasts with the situation for Ly49, where in both adult (32) and fetal NK clones (17) expression appears to be controlled predominantly or exclusively at the transcriptional level. However, it is in agreement with a recent report that some γδ T cells contain intracellular but not surface CD94 complexes (33), and suggests that in some cases surface expression of CD94/NKG2 complexes may be controlled at the level of assembly and transport to the cell surface. It has been established that for CD94/NKG2C complexes, association with DAP12 is required for surface expression (34), but whether additional components are required for surface expression of CD94/NKG2A complexes is less clear. Other explanations for the

finding that some clones contain mRNA for CD94 and NKG2 yet lack Qa1R would be that the CD94 and NKG2 messages are being expressed in a mutually exclusive manner among the individual cells of such clones, or that alternatively spliced forms of CD94 and NKG2 molecules (see below) interfere with the expression of Qa1R.

RT-PCR analysis revealed that all of the fetal NK clones examined contained mRNA for the inhibitory NKG2A molecule, the noninhibitory NKG2C molecule, and for the distantly related inhibitory NKG2D molecule. Again, further study will be needed to establish whether these different molecules can be simultaneously expressed by individual cells within clones. In humans, it appears that within the mature NK cell population there are cells that express either NKG2A or NKG2C or both (35, 36). Surprisingly, in both adult and fetal mouse NK cells we were unable to detect expression of NKG2B, a putative alternately spliced form of NKG2A that lacks the stalk region in the extracellular domain. Instead, all clones and lines examined, including adult NK cells, were found to contain what appears to be another alternately spliced form of NKG2A that lacks the transmembrane domain. Based on current knowledge of the translocation of type II proteins, it would seem that a molecule of this type could not leave the cytosol and hence would be nonfunctional. Curiously, though, we have found that adult mouse NK cells contain substantial quantities of alternatively spliced mRNA that would encode Ly49 molecules lacking transmembrane domains (S. Shrestha et al., unpublished data), suggesting that the production of molecules of this type is of some significance.

Another very unexpected finding was that Qa1R expression was strongly regulated by IL-4. When present during the secondary phase of culture in IL-2, IL-4 completely inhibited the acquisition of Qa1R by developing NK cells yet had no effect on the acquisition of NK1.1. Although the growth of NK cells is eventually inhibited by IL-4 (15), the presence of IL-4 during the first few days of culture in IL-2 has no obvious toxic or growth inhibitory effects. It therefore seems likely that IL-4 acts by preventing the de novo expression of Qa1R. Cells that have been exposed to IL-4 during the first 3 days of culture in IL-2 show no tendency to acquire Qa1R when transferred to IL-4-free medium indicating that the effect of IL-4 is largely irreversible, and providing further support for the view that the switching on of Qa1R expression is restricted to a critical time-limited period early in NK development. Remarkably, the effect of IL-4 itself is not confined to this period since, even after 6 days of culture in IL-2, the addition of IL-4 could rapidly reverse the expression of Qa1R (data not shown). Paradoxically, although developing NK cells eventually become susceptible to growth inhibitory effects of high dose IL-4, the long-term growth of fetal NK cells is enhanced by low dose IL-4 (21). It is possible that our use of low doses of IL-4 for this purpose explains why most of our long-term lines and clones lack Qa1R.

The findings reported here concerning the expression and regulation of class I receptors on fetal NK cells go a considerable way toward explaining the lytic specificity of these cells. We previously found that, in contrast to adult NK cells, most preparations of fetal thymus-derived NK cells lysed wild-type blast cells almost as well as class I-deficient blast cells (17). The present finding, that only a minority of the fetal NK cells that develop under the culture conditions used expresses Qa1R, implies that it was these cells that were responsible for the limited discrimination observed, and that the majority of fetal NK cells, due to their lack of receptors for both Qa1-Qdm and classical class I molecules, were unable to discriminate between wild-type and class I-deficient blasts. This hypothesis was directly confirmed by testing purified $Qa1R^-$ and $Qa1R^+$ fetal NK cells and clones. Although we cannot formally exclude the possibility that Qa1R merely act as markers for other class I receptors, strong supporting evidence that Qa1R are directly involved was provided by the observations that 1) L cells expressing a $Qa1/D^b$ hybrid molecule, and 2) T2-Qa1 cells incubated with Qdm peptide, were selectively resistant to $Qa1R^+$ but not $Qa1R^-$ fetal cells. This finding also implies that, as is the case for adult NK cells (19), most $Qa1R^+$ fetal NK cells receive net inhibitory signals following interaction with ligand-expressing cells. Given the stochastic nature of Qa1R expression on NK cells, we suspect that the fetal NK line 1270 that was found to discriminate efficiently between wild-type and class I-deficient blasts in our earlier study (17) contained an unusually large proportion of $Qa1R^+$ cells. Likewise, reports that other populations of Ly49-deficient NK cells can discriminate relatively efficiently between wild-type and class I-deficient blasts (37, 38) may be explained by the different culture conditions or sources of cells used resulting in a higher proportion of the cells acquiring Qa1R.

What is clearly not explained by the class I receptor data we have obtained is the striking ability of fetal NK cells to discriminate between class I-deficient and wild-type EL4 and RMA cells. The fact that these are both tumor cells and are syngeneic to the effector cells is intriguing, but the observation that fetal NK cells efficiently kill a wide variety of other tumor cells, including syngeneic C1498 cells (31), and that both fetal and adult NK cells derived from allogeneic mice can discriminate between wild-type and class I-deficient forms of these cells (unpublished data), argues against either property being relevant. A rational explanation of the results would be that wild-type EL4 and RMA cells express one or more nonclassical class I molecules that are β_2m- and TAP-dependent and are not widely expressed on other tumor cells or blast cells. A potential candidate for such a molecule is CD1, which, although not generally TAP-dependent (39), can associate with peptides (40, 41), and has been reported to protect cells from NK lysis (30). However, the finding that both RMA and RMA/S express similar levels of CD1, and that RMA/S cells overexpressing CD1 do not become resistant to lysis by fetal NK cells, rules out a role for CD1 unless the structure recognized by NK cells on EL4/RMA cells is CD1 loaded with TAP-dependent peptides. In our hands, adult NK cells could also not distinguish between untransfected and CD1-transfected RMA/S cells. The reason for the difference between our results and those of others (30) is currently unclear.

If the resistance of EL4 and RMA cells to lysis by NK cells is due to their expression of a class I molecule, most or all fetal NK cells, and probably also adult NK cells, must express an inhibitory receptor for this molecule. The finding of a variant fetal NK cell line, 1080, that can no longer discriminate between wild-type and class I-deficient EL4/RMA cells, and the discovery that this line has lost expression of at least three members of the NKG2 family, provides strong support for the hypothesis that NK cells express novel class I receptors, and suggests that these may involve members of the NKG2 family. Given the current evidence that several members of this family recognize Qa1/HLA-E (7, 19), an intriguing possibility is that the protective element on EL4/RMA cells is a form of Qa1 that associates with non-Qdm peptides (42) or with other proteins (43, 44). It also strongly suggests that the defect in fetal NK line 1080 resides in a previously unsuspected factor that is selectively required for the transcription of NKG2 genes. This factor clearly does not affect transcription of the closely related CD94 molecule, implying that CD94 molecules may have a biological role that is independent of their association with members of the NKG2 family.

Finally, we reported previously that, although fetal NK cells lack expression of most Ly49 molecules, they express high levels of mRNA for the putatively inhibitory Ly49 molecule, Ly49E (17). We report here that Ly49E mRNA is expressed in 11/11 fetal

NK clones examined, a pattern quite distinct from that found for other Ly49 molecules (32, 45). Whether it is expressed in all individual NK cells present within a clone and whether Ly49E protein is expressed on the cell surface requires further investigation. If these assumptions are correct, the presence of Ly49E mRNA in Qa1R$^-$ NK cells that readily lyse normal blasts would indicate that Ly49E is not an inhibitory receptor for any of the class I molecules expressed on blast cells of C57 mice. Similarly, the presence of Ly49E mRNA in the 1080 line would indicate that Ly49E does not recognize the putative protective class I molecule expressed on EL4/RMA cells unless it forms a heterodimeric structure with an NKG2 molecule. The possibility that the natural ligand for Ly49E may not be a class I molecule should therefore be considered seriously.

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