Fetal Liver T Cell Receptor γ/δ^+ T Cells as Cytotoxic T Lymphocytes Specific for Maternal Alloantigens

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Summary

We have established fetal liver-derived T cell receptor (TCR)γ/δ^+, CD3$^+$ T cell lines that are cytotoxic for maternal T cells. Fetal liver-derived lymphoid progenitors yielded predominantly TCR-γ/δ^+ cell clusters when cultured on fetal bone marrow-derived stromal cells in the presence of a cytokine cocktail under magnetic force. These tightly adherent clusters were cloned by limiting dilution and the resulting cell lines analyzed for phenotype and function. Six of eight TCR-γ/δ lines from 8–9.5-wk gestation fetuses were V$\delta2^+$ as compared with zero of eight lines from later stages of gestation (10 and 15 wk), where all the lines were V$\delta1^+$. In cytotoxicity assays, these TCR-γ/δ^+, CD3$^+$, CD4$^-$, and CD8$^+$ or CD8$^-$ long-term cultured lymphoid cells (LLC) were killer cells active against the class I antigens on maternal T cells. Of the cell lines, the CD8$^+$ TCR-γ/δ^+ LLC had the highest levels of killer activity. Thus fetal liver TCR-γ/δ^+ T cells may play a crucial role in protection against invading maternal T cells generated in the feto-maternal interaction.

The development and function of fetal lymphocytes is of particular interest in terms of feto-maternal immunologic interactions. In recent years, we have found cytotoxic fetal antibodies that react against maternal alloreactive T cells (1–3). We have further demonstrated that fetal CTLs can be generated by the stimulation of maternal alloreactive T cells (3).

In recent years, two types of heterodimeric forms of TCR have been defined. TCR-α/β and TCR-γ/δ (4–6). Although the precise function of TCR-γ/δ^+ cells is not yet known, TCR-γ/δ^+ cells can recognize Ag in a MHC-restricted manner (7), undergo TCR-mediated cell activation (8), and participate in immune surveillance at the epithelial cell surface (9).

CD7$^+$, CD4$^-$, CD8$^-$, and CD3$^-$ T cell precursors enter the human thymus at 7–8 wk of gestation and begin to express CD2 Ag intrathymically (10, 11). Expression of CD2 molecules by immature thymocytes before expression of surface CD3 molecules suggests the involvement of CD2 molecules in triggering immature TCR-negative thymocytes (12, 13). The TCR-β and -δ epitopes are first expressed at 9.5 wk of gestation. TCR-δ^+ cells comprise only a minority of fetal thymocytes throughout ontogeny with the highest percentage (11%) seen at 9.5 wk of gestation. In contrast, the percentage

of TCR-β^+ cells (surface and cytoplasmic) increases from 0% at 8.5 wk to 91% at 15 wk of gestation (14).

A variety of cytokines such as IL-2 (15), IL-3 (16, 17), and IL-5 (18–20) are involved in lymphoid cell development. In addition, stromal cells are essential for the growth and differentiation of lymphoid cells (21). In our present study, we have developed a magnetic culture system that can promote the growth of human lymphoid precursors in the presence of several such cytokines and bone marrow-derived stromal cells (BMST)[1]. This culture system has made it possible to grow fetal lymphoid precursors for 6 mo or longer and obtain long-term cultured lymphoid cells (LLC) derived from a single cluster. We have determined cellular characteristics and functional properties of both TCR-γ/δ^+ and TCR-α/β^+ fetal liver-derived T-LLC.

Materials and Methods

Preparation of Fetal Samples. Fetal bone marrow and liver were obtained from the Department of Clinical Pathology of Shinshu

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[1] *Abbreviations used in this paper:* BMST, bone marrow-derived stromal cells; LLC, long-term cultured lymphoid cells; TLFA, T lymphocytotoxic human fetal antibodies.

University as discarded tissues from either elective first trimester abortions or at the time of surgery for ectopic pregnancy. The gestational age ranged from 8 to 15 wk as determined by crown–rump length and menstrual records. The fetal specimens were placed into heparinized cold HBSS, and vigorously mixed with a rubber policeman and through a metal mesh. The bone marrow cell suspensions were diluted with heparinized cold HBSS, layered over 40% Percoll density gradients, and centrifuged at 2,000 g for 20 min at 4°C. Cells of the 40% fraction were washed twice with cold HBSS and used as sources of BMST. Stromal actin was detected with the CGA-7 mAb (Enzo Diagnostic, New York) and the antigen-positive cells were treated as described below for the expansion of BMST. The liver cell suspensions were layered over a 60% Percoll density gradient, centrifuged, and then cells from the 60% fraction were washed with cold HBSS. Contaminating mature lymphocytes were removed by treating 2×10^6 cells with 2×10^7 anti-CD2 and anti-CD19 coated Dynabeads (Dynal A. S., Oslo, Norway). CD34$^+$ cells were then isolated using anti-CD34 coated Dynabeads. The purified CD34$^+$ cells were collected, washed with cold HBSS, and used as sources of lymphoid progenitors.

Magnetic Cell Culture System. A newly designed magnetic culture system was employed here. The system consisted of magnetic alloy tablets (400–1,800 gauss). To grow original cytokine-induced BMST, cells of the 40% Percoll fraction (1–2 $\times 10^4$/ml) were cultured in small magnetic culture dishes (1,800 gauss) in 5% CO_2 in air at 37°C. The basic culture medium contained 10% FCS (Flow Laboratories, Rockville, MD), 2 ng/ml basic bovine fibroblast growth factor (FGF) (Sigma Chemical Co., St. Louis, MO), 50 ng/ml human rGM-CSF (Genzyme Corp., Boston, MA), 2 U/ml rIL-2 (Takeda Pharmaceutical Co., Osaka, Japan), and 20 ng/ml human rIL-5 (Santory Co., Osaka, Japan). After 2 wk, BMST were removed from the plastic surface. The BMST in suspension were again loaded on 40% Percoll gradients, and were purified by collecting cells from the upper fraction. To obtain sufficient numbers of cells, the purified BMST (10^4/ml) were cultured for another 4 wk (6 wk in total) in the presence of the same concentrations of the cytokines in the mild magnetic force (400 gauss) system. About 75% of the culture medium was renewed every week. Supernatants of the BMST culture (BMST-sup) were collected, filtered, and stored for the culture of LLC.

The purified lymphoid progenitors were cultured in the presence of 10% BMST-sup and the cytokine cocktail. After 2 wk, the cells yielded many clusters in the culture system with higher levels of magnetic force (1,800 gauss). Since the cultured cells formed tight cell aggregates, it was difficult to obtain LLC as a single-cell suspension. Cell cloning in a cluster form was performed by the limiting dilution method (0.3 cluster/well) using a feeder layer of preseeded BMST, BMST-sup, and the cytokines in 96-well culture plates. After 2 wk (4 wk after the initial culture), the growing LLC were selected based on positive CD3, WT31, and TCR-δ1 staining. After 4 wk (8 wk of culture in total), TCR-α/β$^+$ T-LLC and TCR-γ/δ$^+$ T-LLC were expanded using 24-well culture plates. They were further cultured in large culture dishes (100 × 15 mm) on BMST in the presence of the cytokines. After 2–4 wk (10–12 wk in total), LLC were collected at 4-d intervals, and they were processed for phenotypic, in situ hybridization, and killer activity assays.

Phenotypic Analysis. Phenotypic analyses of surface antigens were performed on a FACStar® flow cytometry system (Becton Dickinson Monoclonal Center, Mountain View, CA). Immunofluorescence microscopy was also employed for the examination of intracellular immunostaining. A panel of mAbs and polyclonal Abs were used for the analyses: CD3(Leu-4), CD8(Leu-2a), and TCR-α/β (anti-TCR-1: WT31) from Becton Dickinson Monoclonal Center; TCR-γ/δ(δTCS1), TCR-α/β(βF1), TCR-γ/δ(TCS-δ1), δV1(a) (Vδ1), and δV2(a)(Vδ2) from T Cell Science, Inc. (Cambridge, MA). Cells (10^6) were incubated with appropriately diluted mAbs (1:40 for most Abs) for 30 min at 4°C, washed twice, and incubated with a 1:40 dilution of affinity-purified goat anti-mouse IgG-F(ab')$_2$-FITC (Cappel Laboratories, Cochranville, PA). After washing twice, the cells were resuspended in 1-ml PBS with 5% FCS and processed for cytometry. Cytospin slides were fixed and stained with anti-CD3 for 30 min and 5–10 μl of peroxidase-conjugated F(ab')$_2$ fragment of anti-goat, –rabbit, or –mouse Ig(s) on ice for 30 min.

In Situ Hybridization. The in situ hybridization detection system (Bethesda Research Laboratories, Gaithersburg, MD) was employed. The hybridization was performed using a biotin-labeled Cβ1 (22) constant region RNA probe (kindly provided by Dr. T. W. Mak, Toronto, Canada), and a TCR-Cδ1 (23) DNA probe (synthesized by using a DNA synthesizer according to the map).
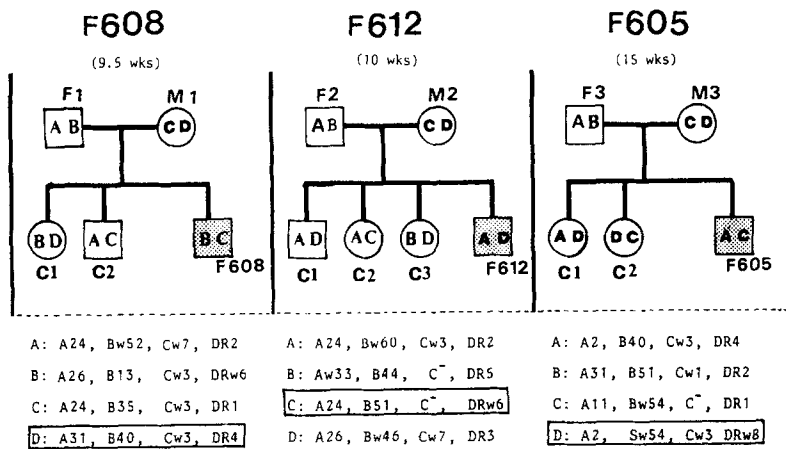
Killer Activity Assay. The killer activity of LLC was evaluated against PHA-activated blastoid T cells (PHA-P: 0.1%) of the mother, father and siblings, according to previous reports (2, 3). For blocking experiments, target cells were treated with anti-HLA panel Abs (Fuji Biomaterials Laboratory, Tokyo, Japan). LLC and target cells were suspended in 0.2 ml of culture medium in each well of microplates in triplicate. The plates were centrifuged at 200 g for 5 min, and incubated for 4 h at 37°C.. From the radioactivity in 0.1 ml of supernatant, percent specific lysis was calculated by the following formula: Percent specific lysis = 100 × (Experimental cpm − medium control cpm)/(Maximum release cpm − medium control cpm). The maximal count was determined by disrupting the target cells in 1.25% saponin solution (Wako Pure Chemicals, Osaka, Japan), and amounted to more than 90% of the control radioactivity taken up by the target cells. The spontaneous ^{51}Cr-release from the target cells was <20% of the maximal release.

Results

Fetal Liver LLC Cultured on BMST. Fig. 1 shows the HLA typing of three family pedigrees. We failed to analyze HLA in one family (F617; 8 wk). To define the involvement of HLA antigens, we selected families that consisted of three to four siblings.

As for the fetal liver TCR-γ/δ LLC grown on fetal BMST, there were 23 clusters/well in F617(8 wk), 184 clusters/well in F608(9.5 wk), 106 clusters/well in F612(10 wk), and 27 clusters/well in F605(15 wk). These data are consistent with the finding that TCR-γ/δ$^+$ cells are prominent at around 10 wk of gestation in the fetal liver (10). The clusters were collected and mixed together. Sufficient numbers of cells were obtainable for the study of their surface antigens, intracytoplasmic antigens, and for in situ hybridization.

Cellular Characteristics of the Fetal LLC. FACS® profiles of TCR-γ/δ–associated antigens on the LLC are shown in Fig. 2. F608(9.5 wk), F612(10 wk), and F605(15 wk) fetal liver LLC were studied for the expression of TCR-γ/δ, Vδ1, and Vδ2. All of the six liver LLC lines were TCR-γ/δ$^+$, consisting of a single population of brightly staining cells. However, there were both dimly and brightly staining Vδ1$^+$ and Vδ2$^+$ cells. As shown in Fig. 3, all of the established

F608
(9.5 wks)

F1 M1
A B C D

B D   A C   B C
C1   C2   F608

A: A24, Bw52, Cw7, DR2
B: A26, B13, Cw3, DRw6
C: A24, B35, Cw3, DR1
D: A31, B40, Cw3, DR4

F612
(10 wks)

F2 M2
AB C D

A D   A C   B D   A D
C1   C2   C3   F612

A: A24, Bw60, Cw3, DR2
B: Aw33, B44, C⁻, DR5
C: A24, B51, C⁻, DRw6
D: A26, Bw46, Cw7, DR3

F605
(15 wks)

F3 M3
AB C D

A D   D C   A C
C1   C2   F605

A: A2, B40, Cw3, DR4
B: A31, B51, Cw1, DR2
C: A11, Bw54, C⁻, DR1
D: A2, Sw54, Cw3 DRw8

Figure 1. HLA typing of three family pedigrees. (*F*) Father. (*M*) Mother. (*Dotted squares*) Fetus. (*Open squares* in the haplotype designations) Unshared haplotypes between the fetus and the mother.

LLC lines showed almost the same pattern of CD3-cyto, CD3, TCR-γ/δ and TCR-Cδ1 positivity. All of them were negative for CD4, TCR-α/β and TCR-Cβ1. There was a wide variation among them in the expression of CD8, Vδ1, and Vδ2. Two of the CD8⁺ LLC clones, one in F612(10 wk) and the other in F605(15 wk), showed potent killer activity as described in the following section. It is interesting that all of the F617(8 wk) LLC lines were Vδ2⁺, but none of them expressed Vδ1. In contrast, all of the F612(10 wk) and F605(15 wk) LLC lines were positive for Vδ1 but negative for Vδ2. Thus, there were no TCR-α/β⁺ T cells in these six LLC lines, and Vδ2⁺ and Vδ1⁺ cells did not coexist in a single line. These data suggest that Vδ2⁺ cells are predominant at around 8 wk of gestation, and Vδ1⁺ cells appear
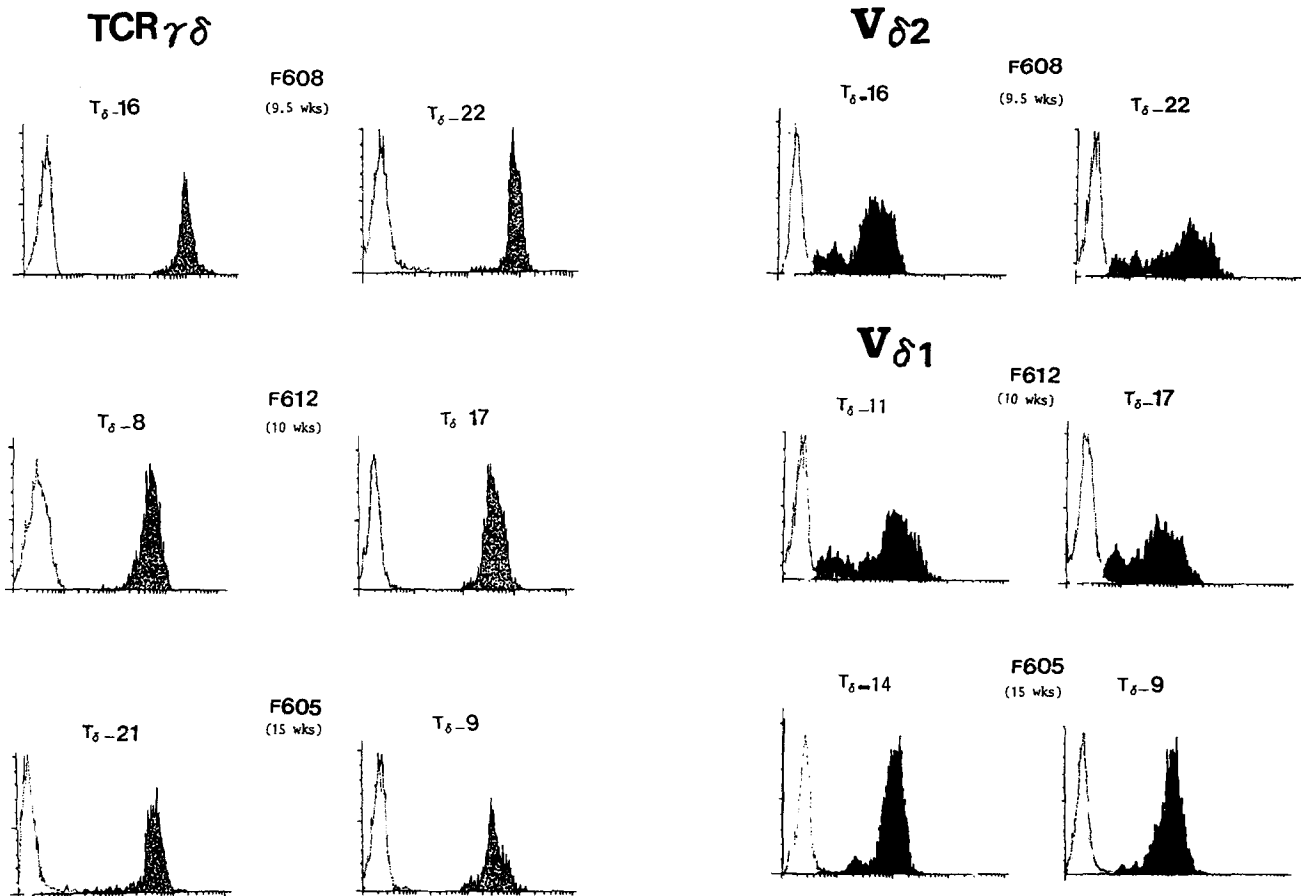
**Figure 2.** FACS® profiles of the established TCR-γ/δ⁺ LLC. (*Dotted portions*) Positive staining with TCR-γ/δ. (*Dotted lines*) Negative control profiles. (*Blackened portions*) Positive Vδ1 and, Vδ2 staining. (*Dotted lines*) Negative control profiles.
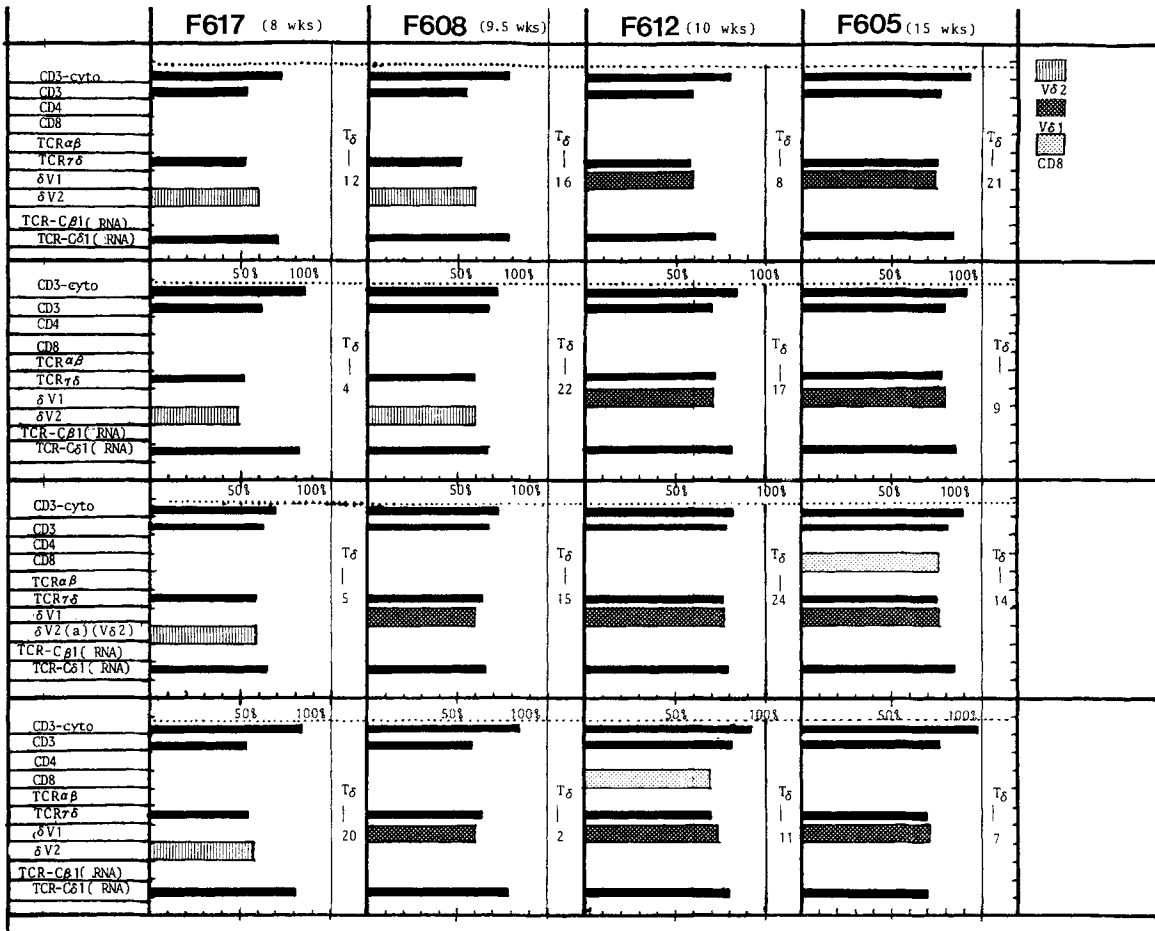
Figure 3. Summary of intracellular, phenotypic, and in situ analyses of the established LLC.
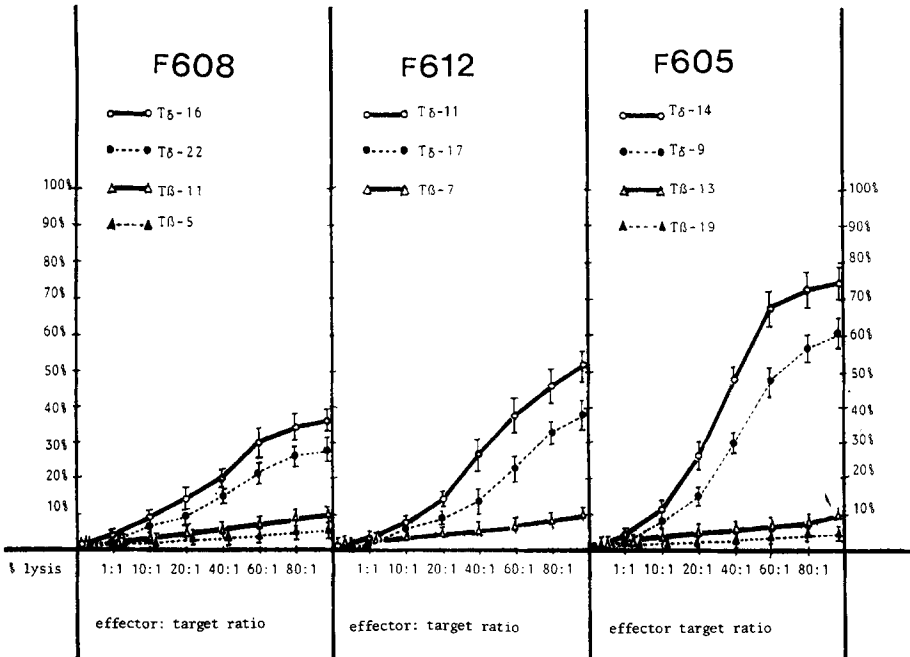
Figure 4. Killer assays of the fetal liver T-LLC. Representative killer activities of Tδ and Tβ T-LLC. Fetal T-LLC were assayed with the respective maternal PHA-activated T cell blasts.
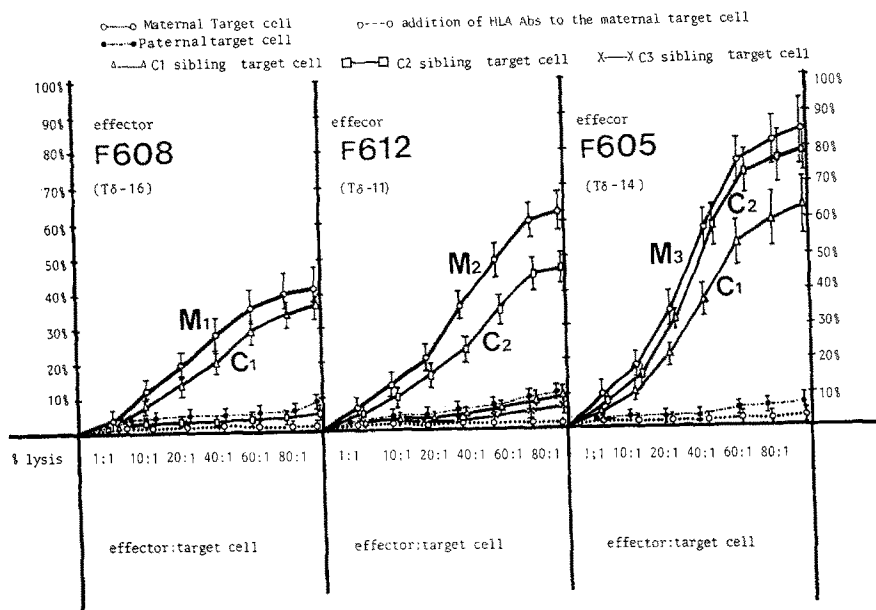
Figure 5. Specificity of the killer activities of fetal liver T-LLC. Killer activities of fetal killer Tδ T-LLC against the maternal, paternal, and sibling PHA-blasts. The maternal Ab blocking was performed by the addition of 1:50 diluted anti-HLA Abs.

in parallel with the development of thymus at around 9 wk of gestation (14).

As shown in Fig. 3, in situ hybridization showed that all of the established LLC lines were positive for TCR-Cδ1 mRNA.

Killer Activity of Cloned Fetal LLC. Fig. 4 shows the cytotoxic potential of fetal liver TCR-α/β⁺ LLC and TCR-γ/δ⁺ LLC against maternal PHA-activated T cells. Six of the TCR-γ/δ⁺ liver T-LLC lines (6/24) from three fetuses had cytotoxic activity against the respective maternal PHA-induced target cells: two of F608 TCR-γ/δ⁺, Vδ2⁺ LLC, two of F612 TCR-γ/δ⁺, Vδ1⁺ LLC, and two of F605 TCR-γ/δ⁺, Vδ1⁺ LLC. The levels of CTL activity varied widely among the lines: the lowest was in Vδ2⁺ F608 Tδ-22, and the highest was in Vδ1⁺ F605 Tδ14. The Vδ2⁺ F608 Tδ16 cells with lower levels of CTL activity were further cultured for 4 wk, and again assayed for CTL activity. The activity levels did not increase (+ 2%). In contrast, the Vδ1⁺ F612 Tδ11 cells showed increased levels of CTL activity (+ 20%) after culture for another 4 wk. These data indicate that CTL activity of Vδ2⁺ LLC is generally lower than that of Vδ1⁺ LLC, and that two of the most active LLC are CD8⁺. Five of TCR-α/β⁺ T-LLC lines (5/24) had no cytotoxic activity against PHA-activated maternal T cells. However, after addition of IFN-γ into these LLC, CTL activity appeared in two of the TCR-α/β⁺ LLC (data not shown).

It was interesting to determine whether or not these TCR-γ/δ⁺ T-LLC had specificity for the maternal HLA antigens. For this purpose, three TCR-γ/δ⁺ T-LLC lines with high levels of CTL activity were selected, and their CTL specificities were analyzed using PHA-activated T cells of the mother, father, and siblings as targets (Fig. 5). F608(HLA-A, -B, and -C) apparently recognizes the maternal D haplotype, since specific cytotoxicity was observed with maternal(HLA: C and D) (Fig. 1) and sibling(C1; HLA: B and D) targets, but not

with target cells from another sibling(C2; HLA: A and C) or the father (HLA: A and B). Similarly, F612 recognizes the maternal C haplotype, and F605 recognizes the maternal D haplotype in the respective families (Figs. 1 and 5). In each case, the maternal haplotype recognized represents an alloantigen for the proband, from which the fetal liver LLC line was derived. In the next experiment, the CTL activity of the T-LLC against the maternal T cells was shown to be completely blocked by the addition of antimaternal HLA-A and -B Abs (Fig. 5). These data suggest that fetal liver TCR-γ/δ⁺ T-LLC are specific for maternal T cells, and that CD8⁺ lines have high levels of the killer activity.

Discussion

Several mAbs have been developed to identify TCR-γ/δ expressing cells (24, 25). Some mAbs used in this study, such as δV1(a) and δV2(a), can recognize variable gene-encoded epitopes and delineate subsets of TCR-γ/δ cells. Other mAbs, such as TCR-δ1, are reactive with all TCR-γ/δ cells, which suggests that they are directed against a C-encoded framework region. The availability of these mAbs has recently allowed the phenotype and tissue distribution of TCR-γ/δ cells to be defined. In humans, about 4% of CD3⁺ cells express TCR-γ/δ in the fetal and postnatal thymus, peripheral blood, and lymphoid organs (24, 25). The maximum percentage of TCR-γ/δ⁺, CD3⁺ cells, about 10%, can be found in the fetal thymus at 9 wk of gestation (14). TCR-γ/δ⁺ cells can recognize Ag in a MHC-restricted manner (7), undergo TCR-mediated cell activation (8), and participate in immune surveillance at epithelial cell surfaces (9). However, the function of fetal TCR-γ/δ⁺, CD3⁺ cells still remains to be examined because of the difficulty of obtaining sufficient numbers of such cells from the early human fetal lymphoid tissues. To overcome this problem, we have used a magnetic culture system for expanding the original cells.

In recent years, we have presented one of the possible mech-

anisms of fetal protection against maternal immunologic attack, in which T lymphocytotoxic human fetal antibodies (TLFA) act to eliminate alloreactive maternal T cells. We identified two types of Abs reactive with the T and killer T cells in maternal-fetal mixed lymphocyte cultures (2, 3). These two Ab specificities are closely associated with MHC class I antigens. However, the mechanism for protecting the fetus from maternal immunologic rejection is probably multifactorial. We have used X-chromosomal analysis in a male fetus to demonstrate that maternal T cells can attack the fetus at the initiation of gestation. In the previous studies (1-3) however, we failed to expand fetal killer T cells that could destroy the invading maternal T cells.

In the present study, we have established fetal TCR-γ/δ^+ T cell lines and have examined their cytotoxic potential. The cytotoxic assays demonstrate that fetal liver TCR-γ/δ^+ cells can be killer T cells for the maternal T cells, and the targets are maternal alloantigens (probably class I). A straightforward possible mechanism for eliciting fetal T-LLC against the maternal immunologic attacks can be envisioned. If the haplotypes of the family members are represented as a father (a, b), a mother (c, d), and a fetus (b, d) then the maternal

T cells share HLA molecules (d) with fetal determinants. However, fetal T-LLC specific for (c) can react with the invading maternal T cells. Actually, in our cytotoxic assay, three of the fetal TCR-γ/δ^+ T-LLC clones showed specificity for the maternal (c) determinants.

It has been reported that pre-T(CD2$^+$, cyto-CD3$^+$, CD7$^+$) cells migrate into the thymus where these immature lymphocytes mature into Vδ1$^+$ cells, and then into Vδ2$^+$ cells (10, 11, 26, 27). Vδ2$^+$ cells then disperse into lymphoid organs and finally appear in the peripheral blood. In this respect, all of the F608(8 wk) liver T-LLC consisted of Vδ2$^+$ cells in our study. The appearance of Vδ2$^+$ cells at 8 wk of gestation in fetal liver before the development of the thymus at around 9 wk of gestation (14), suggests that the liver may be another maturation organ for Vδ2$^+$ cells.

The mother obviously recognizes the fetus as nonself, and the fetus may protect itself from the maternal immunologic attack by eliciting TLFA as a humoral factor, and TCR-γ/δ^+ T cells as a cellular factor. It is also important to take into account the influence of various hormones and other barrier mechanisms of the placenta, as well as immunologic mechanisms in the feto-maternal interaction.

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