T lymphocytes recognize antigen using the TCR, a disulphide-linked heterodimer closely associated with a nonpolymorphic polypeptide complex on the cell surface termed CD3 (1-3). The vast majority of mature T cells in peripheral blood and lymphoid organs use the TCR-a/b, while a minor (1-10%) subpopulation have been shown to express TCR-y/d (4-7). Recently, the finding that both murine Thy-1+ dendritic epidermal cells (8) and intestinal intraepithelial lymphocytes (9) express mainly the y,d receptor led some investigators to propose that T cells carrying this complex could be involved in surveillance of epithelial cell surfaces (10). Interestingly, epidermal cells lack the CD4 and CD8 molecules found on virtually all CD3 a,b T cells, while intestinal epithelial cells are exclusively CD8+ , roughly half of them lacking the Thy.1 marker. Since during ontogeny, cells bearing the y,d receptor appear before those bearing a,b (10), we decided to explore their presence throughout human T cell development. We now report that a considerable proportion of fetal liver T cells in 20-wk-old human fetuses are able to grow in IL-2 and express the y,d receptor. One striking feature of these cells, besides their anatomical location, is the fact that up to 20% of them express the CD4 marker not previously described on the y,d T cells. Analysis of this population at the clonal level reveals that, in contrast with the other CD4 a,b T cell clones and with y,d double-negative (DN) cells, y,d CD4+ cells do not produce IL-2 and are devoid of any cytolytic activity. Thus, they display different properties than either CD4+ a,b T cells or y,d DN cells, suggesting that CD4+ y,d T cells are an ontogenically restricted population expressed at early stages of development, with unique features in the T cell compartment.

Materials and Methods

Isolation of Lymphocyte Populations. Cell suspensions from liver were obtained by mechanical disruption of the tissue fragments and purified by Ficoll-Hypaque centrifugation as described (11).

Monoclonal Antibodies. Anti-CD2 (Leu 5) and anti-CD4 (Leu 3a) were obtained from Becton-Dickinson & Co. (Mountain View, CA); anti-CD3 (SPYT3b) was the kind gift of Dr. J. De Vries (12); anti-CD8 (B9.4) was kindly provided by Dr. C. Mawas (13); anti-(a,b)
TCR (WT31) was obtained from Sanbio (Holland) (14) and Becton Dickinson & Co; anti-TCR-γδ (11F2) was kindly donated by Dr. J. Borst (15); and anti-Tac (H108) was the generous gift of M. L. Botet (16).

**Quantitative Flow Cytometry.** Quantitation of the surface staining of 10^6 viable cells was performed as indicated (11). Reagents included the indicated mAbs. Flow cytometry analyses were performed in a Coulter Profile Analyzer (Coulter, Hialeah, FL). The data were analyzed for representation using the FlowSys program developed by L. Pezzi.

**Cell Cultures.** Fetal liver cells were maintained in vitro under continuous stimulation with a mixture of allogeneic irradiated peripheral blood lymphocytes and irradiated lymphoblastoid cell lines in the presence of rIL-2 and PHA. Limiting dilution analysis under these conditions has shown that one out of one T cell is able to grow (17 and unpublished results).

**Cytotoxicity Assay.** Cytolytic activity was analyzed against ^51Cr-labeled L615 LCL cells, either uncoated or coated with PHA, as described (17).

Results and Discussion

Human fetal samples were obtained from legal therapeutic abortion by cesarean intervention, and cell suspensions from liver (FL) were made by mechanical disruption of the tissue fragments, as described (11). After purification on Ficoll-Hypaque gradients, cells were routinely analyzed by fluorescence flow cytometry for the expression of CD45 antigen, expressed only on cells of hematopoietic lineages and other T cell markers such as CD3, CD2, CD1, CD4, and CD8. Of 28% CD45^+ FL cells, 6% were CD2^+, 4% were CD3^+, and 3% were Bl, in contrast with spleen and thymus, in which values equivalent to levels in newborns were found (Aparicio, P., J. M. Alonso, Miguel A. R. Marcos, and C. Martinez-A., manuscript in preparation). These results indicate the presence of phenotypically mature T cells in FL.

To determine the composition of the CD3-associated TCR of FL T cells, T cell populations were expanded upon in vitro stimulation with a mixture of lymphoblastoid cell lines (LCL) and PBL, in the presence of exogenous rIL-2 (17). Double-color fluorescence staining and flow cytometry analyses were carried out on the expanded population. Fig. 1 shows the distribution of α,β- and γ,δ-bearing cells among the CD3^+ T cells. We found that up to 32% of total T cells express the γ,δ complex, in contrast with 63% of cells using the α,β complex. Of great interest is the distribution of the different populations of γ,δ^+ cells on the basis of the expression of the CD4 or CD8 antigens. In contrast with previous studies, γ,δ cells bearing the CD4 molecule represent a considerable proportion (25% of the entire γ,δ population), while the previously described populations, that is DN γ,δ and CD8^+ γ,δ cells, also present, account for 50 and 25%, respectively. Also worthy of mention is the distribution of CD2^+ cells: up to 15% of cells are CD2^+ CD3^+, phenotype previously ascribed to T cell precursors (17; Alonso, J. M., P. Aparicio, and C. Martinez-A. manuscript preparation).

Previous studies have shown that γ,δ T cells exhibit NK activity (18), conventional cytotoxic T cell activity (19), and spontaneous cytotoxicity (20), indicating therefore that they are implicated in surveillance (10). To assess the function of the new γ,δ CD4^+ cells, we isolated a number of lines by cloning fetal liver cells in limiting dilution, in the presence of phytohemagglutinin (PHA), under in vitro conditions that allowed the growth of one out of one T cell. As shown in Fig. 2, a representative CD2^+ clone was analyzed that expressed and maintained, upon continuous in vitro stimulation, the γ,δ CD3 complex as well as the coreceptor molecule CD4, detected by both anti-δ (15) and anti-γ (kindly donated by M. Brenner) antibodies.
FIGURE 1. Two-color immunofluorescence flow cytometry analyses for coordinate expression of CD3, CD4, CD8 TCR-α/β and TCR-γ/δ in fetal liver cells. Immunofluorescence studies were carried out in single cell suspensions after 15 d in culture. Fetal liver cells were stained in sequential steps by the use of mAbs of predefined specificity, either unlabeled or conjugated with phycoerythrin where indicated, followed by FITC-conjugated goat anti-mouse isotype-specific second-step reagents (Southern Biotechnology, Birmingham, AL). Background values were obtained using isotype-matched, irrelevant antibodies and their respective second antibodies. Percentages of positive cells in each quadrant are indicated for clarity. Three-parameter data were obtained using logarithmic amplification of forward and side-scatter and logarithmic amplification of fluorescence emission. Each profile represents fluorescence data from list mode files after gating out dead cells on the basis of forward and side-scatter.

The γ,δ CD4+ line was expanded and subcloned in vitro (not shown) by stimulation with a mixture of LCL and PBL in the presence of exogenous rIL-2 and PHA. Functional analyses were carried out as described in Fig. 3. Comparative studies between γ,δ CD4+ cells and α,β CD4+ cells were done to assess functional responses...
CD8− (□) and γ,δ CD4+ CD8− (○) T cell clones as effector cells. Spontaneous release was <15%. Non-significant lysis of LG15 was obtained in the absence of the lectin. (B) Expression of the p55 chain of the IL-2R (Tac) was measured after stimulation, as shown below, of the γ,δ CD4+ CD8− T cell clone (Δ) or the two α,β CD4+ CD8− T cell clones (O, □). 24 h after stimulation, cells were stained using biotin-conjugated anti-Tac (H108, IL-2R) antibody followed by phycoerythrin-labeled streptavidin as described in Materials and Methods. Staining performed with αCD4 antibodies shows that >95% of the cells are CD4+. The percentage of positive cells was determined after incubation of 4 × 10^3 cells/well with the indicated concentration of αCD3 antibodies fixed on solid phases.

to anti-CD3 antibodies, measured by IL-2R expression, IL-2 secretion, and cytotoxic activity in a redirected-lysis assay, using PHA as a glue, in which a γ,δ DN T clone was also tested (21). As shown in Fig. 3, γ,δ CD4+ T cells are devoid of any cytotoxic activity, like the clone shown here as a negative control that expresses α,β CD4+ CD8− and in contrast with γ,δ CD4+ CD8−, implying a different activity in the T cell subpopulations tested (Fig. 3 A). Analyses of the T cell activation induced by αCD3 antibodies revealed a differential effect of the γ,δ CD4+ cells when compared with the α,β CD4+ T lymphocytes. As shown in Fig. 3 B, IL-2R expression correlates with increasing concentrations of αCD3 antibodies in both T cell subpopulations analyzed. However, maximal induction of the γ,δ T cell clone requires 10–50 times higher antibody concentration. This effect, which can also be observed by measuring clonal expansion, is presently under study and might be due to the different patterns of glycosylation found in the γ-CD3 protein (not shown).

More importantly, these cells are also different from the α,β CD4+ cells in that they produce very little if any IL-2, explaining the need for continuous addition of exogenous IL-2 for the cell line to grow. The functional differences of γ,δ CD4, as compared with γ,δ DN cells and their α,β counterparts, might reveal that γ,δ CD4+ T cell populations probably represent cells with properties yet to be characterized. It is not at all clear whether their differential function derives from the fact that their receptors are encoded by a different set of genes displaying a different pattern of recognition or from the fact that they represent a different cell lineage.

In light of the heterogeneity described for the α,β T cells, we can not be certain as to whether the characteristics described here would apply to all γ,δ CD4+ T cells. However, recent results indeed support this possibility.

Since γ,δ CD4+ T cells could not be found in the thymus, where the γ,δ cell population expanded under the same culture conditions represents <5% of the total T cells, it could be speculated that they are the product of locally differentiated precursors.
most likely occurring in the absence of thymic imprinting. Furthermore, their phenotypic and ontogenic appearance and their functional differences probably imply that they play an important, as yet uncharacterized role in the growth and differentiation of the rest of the T cell population characterized so far.

Finally, although the complete understanding of the overall biological role of the γ,δ T cells will require the identification of their natural ligand, the existence of γ,δ CD4+ cells poses new questions in T cell development. Thus, previously, the existence of DN or CD8+ was only considered in connection with speculation on the possibility of recognizing class I MHC antigens, a concept that must be reconsidered now, in the light of our results. It appears, therefore, that there are major differences between γ,δ and α,β populations, not only with respect to the limited diversity generated in γ,δ in comparison with α,β, but also in the function they display.

Summary

Lymphocytes isolated from human fetal liver and expanded in vitro in IL-2-containing media reveal the existence of CD4+ γ,δ T cells. These cells display differential features of double-negative and CD8+ γ,δ T cells as well as of CD4+ α,β T cells. Thus, they fail to lyse targets in lectin-mediated killing assays and to perform classical helper functions. These results add new information necessary for a better understanding of the physiological role of the γ,δ T cells.

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