

IDENTIFICATION OF CD2<sup>-</sup>/CD3<sup>+</sup> T CELLS  
IN FETAL HUMAN TISSUE

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Recent evidence indicates that T11 (CD2), the sheep erythrocyte receptor, is a functionally important molecule on the surface of human T lymphocytes. CD2 binds the cell surface ligand LFA-3 to mediate adhesion between T cells and other cell types (1, 2). In addition, CD2 is the target structure for an "alternative" pathway of T cell activation. Thus, it has been shown that a combination of mAbs against the T11<sub>2</sub> and T11<sub>3</sub> epitopes of CD2 induce IL-2 production and proliferation in purified T cells and T cell clones (3). This antigen-independent mode of T cell triggering appears to be functionally linked to that involving the CD3/TCR molecular complex (4).

According to the generally accepted view, CD2 is the earliest T cell lineage-specific differentiation antigen to appear during thymic ontogeny, and precedes the expression of CD3 (3, 5). This differentiation model does not allow for any significant number of cells with a CD2<sup>-</sup>/3<sup>+</sup> phenotype among immature (or mature) T cells. In line with this prediction, cell surface expression of CD3 has been found to depend on the simultaneous expression of CD2 on a number of  $\gamma$ -radiation-induced variants of the Jurkat T cell clone (6). In contrast to this proposal, however, we now report that significant numbers of CD2<sup>-</sup>/3<sup>+</sup> T cells are readily identified in specimens of fetal human spleen and thymus. In addition, we have established IL-2-dependent T cell clones from fetal human thymus that have maintained a CD2<sup>-</sup>/3<sup>+</sup> phenotype over several months of continuous culture. Therefore, we conclude that cell surface expression of the CD2 differentiation antigen is not necessarily required for the expression of the CD3 molecular complex on fetal human T lymphocytes.

### Materials and Methods

*Cell Preparations.* Frozen specimens of fetal human thymus and spleen obtained between the 20th and 24th week of gestation were kindly provided by Dr. W. Heit, Department of Hematology, University of Ulm. After thawing, cells were centrifuged on Ficoll-Hypaque gradients to remove dead cells and debris. Mononuclear cells (MNC) were analyzed for the presence of CD2<sup>+</sup> and CD3<sup>+</sup> T cells by simultaneous double-color cytofluorometry. 10<sup>6</sup> MNC were incubated for 30 min on ice with FITC-conjugated Leu5b (CD2) mAb and phycoerythrin (PE)-conjugated Leu4 (CD3) mAb (both from Becton Dickinson & Co., Mountain View, CA). After washing with PBS/1% FCS/0.1% sodium azide, cells were analyzed on a cell sorter (Epics V; Coulter Electronics, Hialeah, FL) on the basis of forward angle light scatter and fluorescence intensity.

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*Cell Cloning.* Fetal spleen and thymus MNC were stained with FITC-Leu5b plus PE-Leu4 mAbs as described above. CD2<sup>-</sup>/CD3<sup>+</sup> and CD2<sup>+</sup>/CD3<sup>+</sup> T cell clones were established by sorting stained MNC and delivering single cells of the respective phenotype into wells of 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) with the aid of an Epics V autoclone device. The cultures were supplemented with 10<sup>5</sup> irradiated peripheral blood feeder cells per well, 1 µg/ml PHA-P (Wellcome Diagnostics, Burgwedel, FRG), and 50 U/ml human rIL-2 (Biogen, Geneva, Switzerland). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Growth-positive wells were expanded in rIL-2-containing medium; established T cell clones were restimulated every third week with irradiated EBV-transformed B cell lines and PHA-P, and were further propagated in rIL-2-supplemented medium RPMI 1640 (Biochrom KG, Berlin, FRG) supplemented with 10% heat-inactivated FCS.

*Phenotypic Characterization of T Cell Clones.* The following mAbs were used in direct or indirect staining procedures. Anti-CD2: OKT11 (Ortho Pharmaceutical, Raritan, NJ), Leu5b (Becton Dickinson & Co.), HuLy-m1 (reference 7; Camon, Wiesbaden, FRG), MT 1100 (8); anti-CD3: Leu4 (Becton Dickinson & Co.); anti-TCR-α/β heterodimer: BMA031 (9); anti-CD4: OKT4 (Ortho Pharmaceuticals); anti-CD8: Leu2a (Becton Dickinson & Co.). Rosetting of T cell clones with sheep erythrocytes was performed as follows. 2 × 10<sup>5</sup> cloned T cells were centrifuged with 0.1 ml of a 10% suspension of neuraminidase-treated sheep erythrocytes (E<sub>s</sub>). After incubation for 60 min on ice, the pellet was gently resuspended, and rosette formation was visualized under an inverted microscope.

## Results and Discussion

*Identification of CD2<sup>-</sup>/3<sup>+</sup> T Cells in Fetal Tissue.* We have used simultaneous double-color cytofluorometry to study the expression of CD2 and CD3 antigens on the surface of fetal human spleen and thymus cells. To this end, MNC were stained with FITC-conjugated Leu5b (CD2) and PE-conjugated Leu4 (CD3) mAbs and analyzed on an Epics V cell sorter. In addition to commonly observed phenotypes of CD2<sup>+</sup>/3<sup>+</sup> mature T cells and CD2<sup>+</sup> 3<sup>-</sup> "stage I" T cells (5), varying yet significant numbers of CD2<sup>-</sup>/3<sup>+</sup> cells were detected in the spleen and/or thymus of all seven fetal specimens tested. As reported in Table I, from 1 to 23% of fetal spleen MNC and from 3 to 23% of fetal thymus MNC displayed the CD2<sup>-</sup>/3<sup>+</sup> phenotype. Whereas thymus MNC contained more CD2<sup>-</sup>/3<sup>+</sup> cells than splenic MNC in five of seven cases, the reverse was true for two other specimens (P712, P736) from the 24th week of gestation. A representative three-dimensional fluorescence intensity plot of P736 spleen MNC stained with FITC-Leu5b and PE-Leu4 is shown in Fig. 1. A distinct peak of CD2<sup>-</sup>/3<sup>+</sup> cells is clearly present on the y-axis.

*Phenotypic Characterization of a CD2<sup>-</sup>/3<sup>+</sup> T Cell Clone.* To verify the existence of CD2<sup>-</sup>/3<sup>+</sup> T cells at the clonal level, we have established IL-2-dependent clones with the aid of the Epics V autoclone device. Fetal MNC were stained with FITC-Leu5b and PE-Leu4, and cell sorter gates were set to deposit single cells of either CD2<sup>-</sup>/3<sup>+</sup> or CD2<sup>+</sup>/3<sup>+</sup> phenotype into wells of microtiter plates containing irradiated feeder cells, PHA-P, and rIL-2. Growing clones were expanded over 4 mo in rIL-2-supplemented medium. The phenotypic characterization of two representative clones (both derived from fetal thymus P712) is shown in Fig. 2. Clone D637/2 is CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, TCR-α/β<sup>-</sup>, and CD2<sup>-</sup> as revealed by a panel of mAbs directed against the T11<sub>1</sub> epitope of CD2. In contrast, clone D637/5, selected from a CD2<sup>+</sup>/3<sup>+</sup> progenitor, displays the CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, TCR-α/β<sup>-</sup>, CD2<sup>+</sup> phenotype. In line with the CD2 surface expression, clone D637/5 forms rosettes with sheep erythrocytes, while clone D637/2 does not (Fig. 3). Recently, we found that both

TABLE I  
*Simultaneous Double-Color Analysis of Different Fetal Human Thymus and Spleen Specimens with FITC-labeled Leu5b (CD2) and PE-labeled Leu4 (CD3) mAbs*

Sample	Week of gestation	Organ	Percentage of cells with phenotype:			
			CD2 <sup>-</sup> /3 <sup>+</sup>	CD2 <sup>+</sup> /3 <sup>+</sup>	CD2 <sup>+</sup> /3 <sup>-</sup>	CD2 <sup>-</sup> /3 <sup>-</sup>
P677	22	Spleen	1.2	9.6	4.8	84.4
		Thymus	3.4	45.7	22.6	28.3
P712	24	Spleen	23.5	13.1	2.5	60.9
		Thymus	22.7	29.8	7.3	40.2
P736	24	Spleen	19.6	19.5	3.0	57.9
		Thymus	8.6	46.3	15.3	29.8
P756	20	Spleen	4.3	11.4	9.3	75.0
		Thymus	11.1	61.0	10.6	17.3
P797	22	Spleen	5.1	5.2	2.9	86.8
		Thymus	14.0	28.5	11.0	46.5
P834	ND	Spleen	4.3	5.0	4.0	86.7
		Thymus	16.8	38.6	4.4	40.2
P1295	20	Spleen	2.8	8.4	7.2	81.6
		Thymus	12.9	26.2	11.7	49.1

clones express the TCR  $\gamma$  chain as shown by reactivity with the T $\gamma$  A mAb (10). In addition to clone D637/2, we have established seven clones from two fetal spleen and one fetal thymus samples that have maintained a stable CD2<sup>-</sup>/CD3<sup>+</sup> phenotype over 2–4 mo of continuous culture.

These data clearly demonstrate that significant numbers of CD2<sup>-</sup>/CD3<sup>+</sup> T cells are present in fetal human spleen and thymus obtained between the 20th and 24th week of gestation. It should be stressed that cryopreserved material was used throughout this study to identify CD2<sup>-</sup>/CD3<sup>+</sup> T cells. We cannot completely rule out the possibility that particular cell subsets are preferentially lost or preserved during storage in liquid nitrogen. Therefore, further studies will be conducted to identify CD2<sup>-</sup>/CD3<sup>+</sup> T cells on freshly obtained fetal material. Together with the fact that phenotypically stable clones of CD2<sup>-</sup>/CD3<sup>+</sup> T cells can be established from fetal

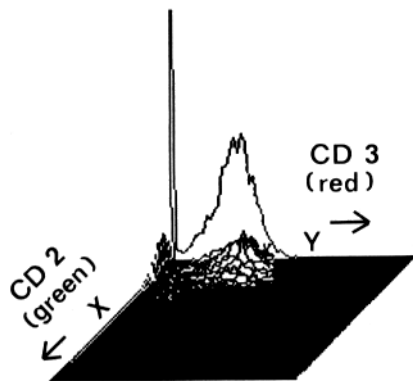


FIGURE 1. Three-dimensional fluorescence intensity plot of CD2 and CD3 expression. Fetal spleen MNC (sample P736) were stained with FITC-Leu5b (CD2; x-axis) and PE-Leu4 (CD3; y-axis) and analyzed on an Epics V cell sorter. A distinct peak of CD2<sup>-</sup>/CD3<sup>+</sup> cells is seen on the y-axis. The distribution of CD2<sup>+</sup> and CD3<sup>+</sup> cells was as follows: 19.6% CD2<sup>-</sup>/3<sup>+</sup>, 19.5% CD2<sup>+</sup>/3<sup>+</sup>, 3.0% CD2<sup>+</sup>/3<sup>-</sup>, and 57.9% CD2<sup>-</sup>/3<sup>-</sup>.

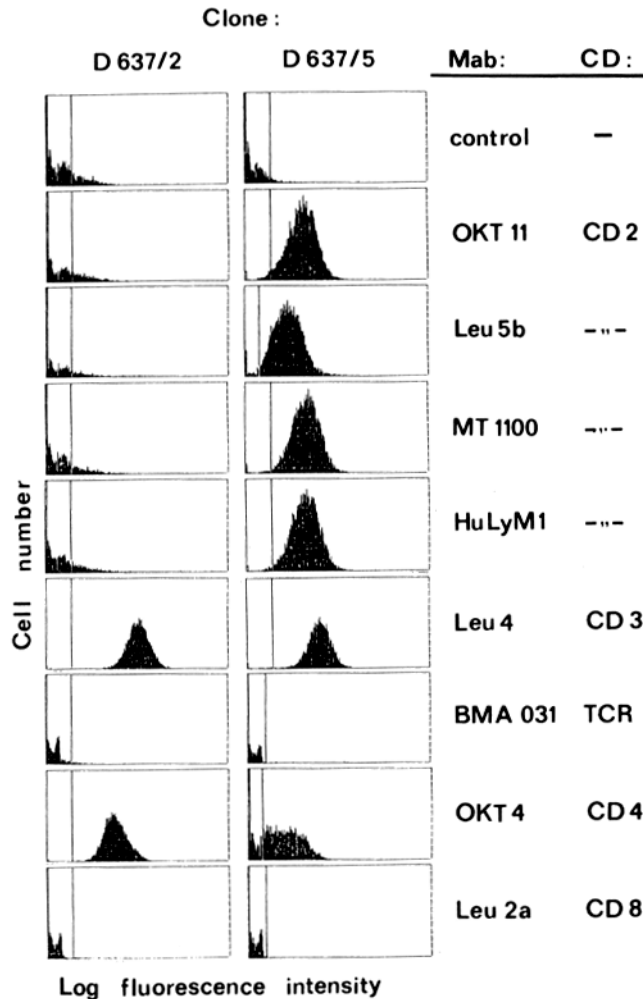
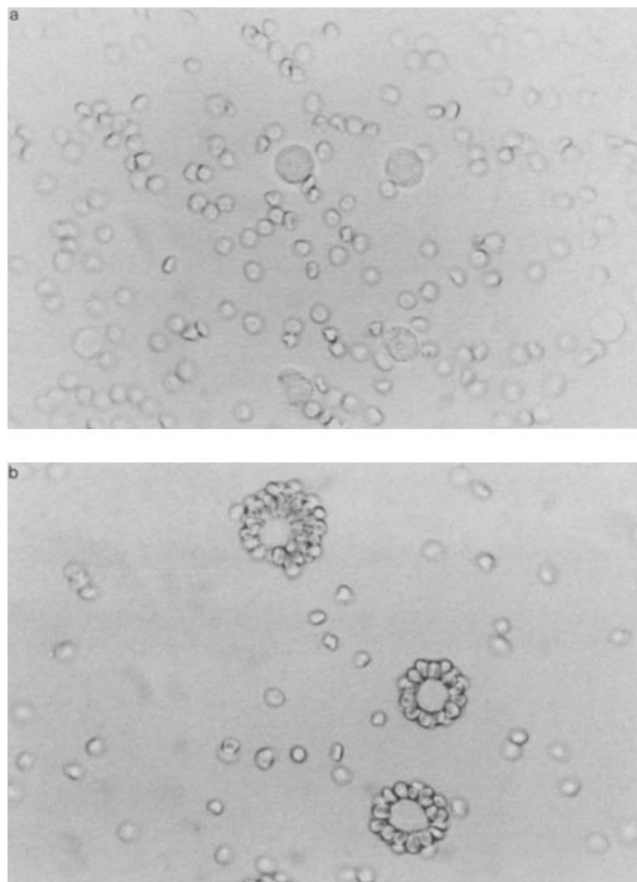


FIGURE 2. Surface marker analysis of IL-2-dependent clones derived from fetal thymus P712. Clone D637/2 was established from CD2<sup>-</sup>/3<sup>+</sup> (Leu5b<sup>-</sup>/Leu4<sup>+</sup>) progenitor, clone D637/5 from a CD2<sup>+</sup>/3<sup>+</sup> (Leu5b<sup>+</sup>/Leu4<sup>+</sup>) progenitor. Cloned cells were stained with fluorochrome-conjugated mAbs (Leu5b-FITC, Leu4-PE, OKT4-FITC, Leu2a-PE), or with unconjugated mAbs (OKT11, MT1100, HuLyM1) and FITC-labeled F(ab)<sub>2</sub> goat anti-mouse Ig as a second antibody, or with biotin-conjugated mAb BMA031 and PE-streptavidin as a second reagent. All samples were analyzed on an Epics V cell sorter.

tissue, our results argue, however, against the view that cell surface expression of CD2 is an absolute prerequisite for the expression of the CD3 molecular complex (5, 6). This conclusion appears to be further supported by a recent study on CD2 mRNA expression during fetal murine thymus ontogeny, in which Owen et al. (11) detected CD3  $\gamma$  chain mRNA in the absence of CD2 mRNA in day 14 fetal thymocytes. In addition, it has recently been found (12) that a significant fraction of TCR- $\gamma$ <sup>+</sup> T cells from one adult individual expressed the CD2<sup>-</sup>/CD3<sup>+</sup> phenotype. The available data thus suggest that a sizable fraction of CD3<sup>+</sup>/TCR- $\gamma$ <sup>+</sup> T cells do not coexpress the CD2 differentiation antigen. It is unknown at present, however, whether the CD2<sup>-</sup>/CD3<sup>+</sup> phenotype is restricted to TCR  $\gamma$  chain-expressing T cells or whether it is also present among "conventional" TCR- $\alpha/\beta$  mature T cells.

In conclusion, we have identified a novel phenotype of CD2<sup>-</sup>/3<sup>+</sup> human T cells. We anticipate that paired clones of CD2<sup>-</sup>/3<sup>+</sup> and CD2<sup>+</sup>/3<sup>+</sup> T cells as established in this study will be helpful in further elucidating the role of the CD2 molecule in



**FIGURE 3.** Sheep erythrocyte rosette formation of cloned fetal T cells. Clone D637/2 (*a*, *left*) and clone D637/5 (*b*, *right*) were incubated with neuraminidase-treated sheep erythrocytes as described under Materials and Methods.

T cell physiology (13-16). In addition, further studies will address the question of whether  $CD2^-/3^+$  cells are also detected among mature peripheral blood T lymphocytes.

### Summary

From 1 to 23% of fetal human spleen or thymus cells (from the 20th to 24th week of gestation) were found to display a previously unrecognized  $CD2^-/CD3^+$  phenotype. IL-2-dependent, long-term clones of  $CD2^-/3^+$  T cells did not react with a panel of anti-CD2 mAbs and did not form rosettes with sheep erythrocytes. These results show that (*a*) significant numbers of  $CD2^-/3^+$  T cells are present in fetal human spleen and/or thymus; and (*b*) in contrast to the widely accepted view, expression of CD2 is not a prerequisite for the expression of the CD3 molecular complex on human T cells.

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