The Expression of T-Cell Receptor-Associated Proteins in Normal and Leukaemic Immature T Cells

D. Campana [1], E. Coustan-Smith [1], L. Wong [2], and G. Janossy [1]

A. Introduction

T lineage cells express different T-cell receptor (TCR) heterodimers, $\alpha\beta$ and $\gamma\delta$, in association with the same set of monomorphic CD3 polypeptides [1–6]. In the post-natal thymus and the peripheral blood of humans [7, 8], rodents [9, 10] and avians [11, 12] TCR$\alpha\beta$ cells are predominant. The distribution of these subpopulations during ontogeny, however, might deviate from this rule. For example, in 12- to 14-day chicken embryos, cells expressing TCR$\gamma\delta$ are higher in number than cells with membrane TCR$\alpha\beta$ [11, 12].

The origin, proliferative activity and expression of CD3 and TCR molecules of the cells which migrate into the thymic epithelial rudiment are not yet fully documented. Several studies have suggested that the rearrangement of the genes encoding for the TCRβ, -γ and -δ chains occurs early in ontogeny, preceding that of the TCRα locus [9, 13–16]. In mice, approximately 75% of thymocytes have rearranged the β chain locus and transcribe the corresponding mRNA at the 17th day of fetal life, when the activation of the TCRα chain gene is initiated [15, 16].

The putative evidence for the rearrangement of the TCR$\gamma\delta$ genes in immature thymocytes derives mainly from the investigation of their configuration in selected CD4$^-$, CD8$^-$ ("double negative") cells, regarded as T-cell precursors [5, 9, 17]. Since such "double negative" thymocytes also include TCR$\gamma\delta^+$ cells (together with TCR-germline non-T cells), the finding of rearranged TCR$\gamma\delta$ loci in such populations is not entirely unexpected, but the suggestion that these clones are derivatives of immature cells is not fully convincing. On the basis of these experiments it has been hypothesized that the rearrangement of TCR$\gamma\delta$ is attempted first and is followed, when not successful, by that of β and α genes. However the possibility that the two lineages follow separate pathways is still open [18]. It is relevant here that the Cδ locus is situated between the Vα and Jα loci [19]; the rearrangement of α genes deletes the Cδ locus, rendering the simultaneous $\alpha\delta$ or successive $\alpha \rightarrow \delta$ expression unlikely.

It is known that once in the thymus, immature T cells actively divide and accumulate CD3 molecules in the cytoplasm [20–22]. This is an early sign of T-cell commitment which is reflected by the consistent expression of cytoplasmic CD3 (cCD3) in thymic acute lymphoblastic leukaemia (T-ALL), the malignant counterpart of early thymic progenitors [21, 22]. It is also known that these malignant cells monoclonally rearrange their TCRβ and TCRγ genes and transcribe TCRβ mRNA [23, 24]. Nevertheless, it has not yet been explored whether such T-ALL blasts are capable of expressing the various TCR-associated chains in their cytoplasm or in their membrane.

Monoclonal antibodies (MAbs) to human TCR proteins have been recently developed. One of these MAbs, βF1, has a framework reactivity against the TCR$\alpha\beta$

[1] Department of Immunology, Royal Free Hospital School of Medicine, London NW3 2QG, UK
[2] Fetal Tissue Bank, Royal Marsden Hospital, London SW3 6JJ

and recognizes the separated β subunit in Western blotting. This antibody does not bind to the surface of viable T cells and requires membrane permeabilization for its use [25]. The MAb βF1 can also be used in an informative combination with WT31, a MAb which recognizes an epitope jointly formed by the assembled α and β chains of the TCR complex [26]. Cells expressing βF1 but no WT31 contain free β chains without membrane TCR. The third reagent, TCRδ-1, identifies one determinant of the human TCRδ chain on the surface of a T-lymphocyte subpopulation [27].

In our study [27a, b], we first applied MAbs to CD3 and TCR chains in double- and triple-colour staining methods in order to investigate the expression of the TCR$\alpha\beta$ and δ proteins during fetal and post-natal T-cell differentiation. Secondly, we used the same reagents to analyse acute leukaemia blasts and compared these findings to the TCR expression in normal immature T cells.

B. Materials and Methods

I. Handling of Samples

Human fetal tissues were obtained following legal termination of pregnancy. The gestational age ranged from 7 to 20 weeks. This was calculated from the foot length compared to a standard curve constructed on the basis of a comparative analysis of this parameter, menstrual records and, when possible, of crown/rump length in 3000 samples studied at the same institution (L. Wong, manuscript in preparation). Infant and adult thymus samples were from patients undergoing cardiac surgery, aged 3–10 years and 17–25 years, respectively. Cell suspensions prepared from parts of the liver and the thymus samples were washed twice in RPMI-1640 with L-glutamine (Gibco, Paisley, UK) and 10% fetal calf serum.

Normal peripheral blood (PB) samples were obtained from healthy volunteers (aged 23–37 years). PB and bone marrow (BM) samples of patients with acute and chronic leukaemia were received by the laboratory for immunological diagnosis. Mononuclear cells from PB, BM and cell suspensions were separated after centrifugation on Ficoll-Hypaque density gradient and washed three times in phosphate-buffered saline (PBS).

II. Staining of Cells

Monoclonal antibodies used in this study are listed in Table 1. Double-colour immunofluorescence (IF) was performed by using second-layer antisera to different mouse Ig isotypes and to Ig of other species. These were conjugated to fluorochromes such as fluorescein isothyocyanate (FITC) and tetramethylrhodamine isothyocyanate (TRITC) or to colloidal gold. Biotin-conjugated βF1 was used with the corresponding second-layer streptavidin (Sigma Chemicals, Poole, UK; cat. no. S. 4762) labelled with TRITC in the laboratory.

Cells at a final concentration of 2×10^7/ml were resuspended in PBS containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA). The viability, assessed with trypan blue, was >95% in the adult and infant samples and >80% in the fetal samples studied. Fifty milliliters of these suspensions was distributed into LP3 tubes (Luckham, West Sussex, UK), and MAbs were added at saturating concentrations ranging from 0.1 µg to 1.0 µg as determined by titration. After 10 min incubation at room temperature cells were washed twice in PBSA and incubated with second-layer antisera for a further 10 min. After two washes in PBSA a small droplet from the pellet was transferred onto a microscope slide, covered with a coverslip and observed on a Zeiss fluorescence microscope equipped with selective filters for FITC and TRITC and a polarizing filter for the detection of colloidal gold [28]. Cells were also studied on an Epics V Cell Sorter (Coulter Electronics, Hialeah, Florida, USA) using the following settings: laser 200 mW at

Table 1. Monoclonal antibodies and heterologous antisera

	CD	Class	Source
Monoclonal antibodies			
βF1	–	IgG1	TCS cat no. TA1051
TCRδ-1	–	IgG1	Dr. M.B. Brenner, Boston, MA, USA
WT31	–	IgG1	BD cat no. 7770
UCHT1	3	IgG1	Dr. P. Beverley, London, UK
T10B9	3	IgM	Prof. J. Thompson, Lexington, KY, USA
MEM57	3	IgG2a	Dr. I. Hilgert, Prague, Czechoslovakia
NA1/34	1	IgG2a	Prof. A. McMichael, Oxford, UK
RFT11	2	IgG1	RFH
RFT1	5	IgG1	RFH
RFT2	7	IgG2a	RFH
RFT4	4	IgG1	RFH
RFT8	8	IgM	RFH
Leu11b	16	IgM	BD cat no. 7530
RFDR1	anti-HLA-DR	IgM	RFH
2D1	45	IgG1	Dr. P. Beverley, London, UK
RFAL3	10	IgM	RFH
RFB7	37	IgM	RFH
RFB4	22	IgG1	RFH
Ki67	–	IgG1	Dakopatts, Denmark, cat no. M722
Heterologous antisera			
Rabbit anti-TdT			SL cat no. CT-004
G-anti-M Ig FITC/TRITC			RFH
G-anti-R IgG FITC			SL cat no. CT-008
G-anti-R Ig TRITC			SL cat no. SBA-4010-03
G-anti-M IgM TRITC			SL cat no. SBA-1020-03
G-anti-M IgG FITC			SL cat no. SBA-1030-02
G-anti-M IgG1 TRITC			SL cat no. SBA-1070-03
G-anti-M IgG2a FITC			SL cat no. SBA-1080-02
G-anti-M IgG gold-conjugated			Janssen, Belgium, cat no. 23.688.20
Streptavidin TRITC			RFH

TCS, T Cell Sciences Inc., Cambridge, MA, USA; BD, Becton Dickinson, Mountain View, CA, USA; RFH, Royal Free Hospital, London, UK; SL, Sera-Lab Ltd., Sussex, UK.

480 nm; photomultiplier tube 700 V; 515 nm dichroic mirror to collect 90° light scatter and 515 nm blocking filter.

In order to visualize intracellular antigens, cytocentrifuge preparations of unlabelled and labelled mononuclear cells were made. For TdT and Ki67 staining cytospins were fixed in cold methanol for 30 min or in a mixture of cold acetone and methanol for 15 min. For visualizing cytoplasmic antigens slides were fixed in acetone for 5–10 min at 20° C. Cytospins and tissue sections were incubated with MAbs for 45 min and washed in PBS. Second-layer antisera were added for the same length of time. After washing in PBS, the slides were mounted in an equal mix of glycerol and PBS.

Double-colour IF with βF1 and another MAb of IgG_1 class (e.g. UCHT1) was performed as follows. First, cells were stained with UCHT1, followed by goat antiserum to mouse Ig-FITC (G-anti-M Ig FITC). Of these cells cytocentrifuge preparations were made. To these normal mouse serum was added in order

to saturate free combining sites of the previous second layer. Finally, the smears were incubated with βF1 conjugated to biotin and streptavidin TRITC. It is known that βF1 labels β chains only in permeabilized cells. In the triple-staining experiments, cell suspensions were first incubated in suspension with WT31 + G-anti-M IgG conjugated to colloidal gold, and cytocentrifuge preparations were made. After fixation in acetone:methanol 1:1 and incubation for 30 min with diluted normal mouse serum, rabbit antiserum to TdT (R-anti-TdT) and βF1-biotin were both added. The second layers were goat antisera to rabbit Ig conjugated to FITC (G-anti-R Ig FITC) and streptavidin TRITC, respectively. Controls, including MAbs of identical subclass (e.g. CD22; IgG_1) were studied in parallel samples in place of WT31 or βF1.

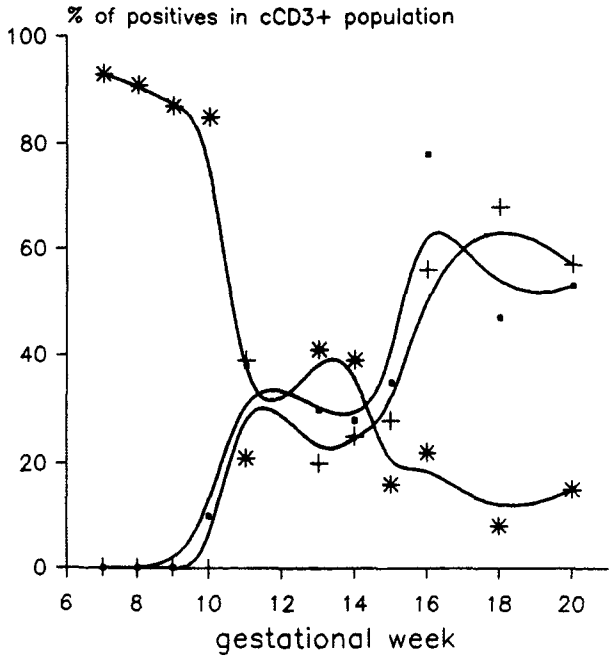

Fig. 1. Phenotypic and proliferative changes of $CD3^+$ cells in the fetal liver during ontogeny. In samples obtained from the 7th to the 9th week of gestation, $cCD3^+$ cells are $mCD3^-$ (+) and $\beta F1^-$ (•). These cells show a high proliferative activity, indicated by Ki67 expression (*). After the 10th week of gestation $mCD3^+$ and $\beta F1^+$ cells appear, and the proliferative activity decreases [27a]

C. Results

I. Phenotype of T Lineage Cells in the Fetal Liver

Fifteen samples of fetal liver of 7–20 weeks gestation were studied [27a]. Lymphoid cells labelled by CD3 (UCHT1) in cytocentrifuge preparations were found in all the samples analysed and represented 0.6%–12% of the mononuclear cells. When the staining was performed in suspensions of viable cells, no membrane CD3 ($mCD3^+$) cells were seen in the liver samples of 7- to 9-week-old fetuses, but a few $mCD3^+$ cells appeared at 10.5 weeks (39% of $cCD3^+$ cells). These cells then persisted in samples from 11- to 20-week-old fetuses. Nevertheless, the percentages of cells labelled by UCHT1 in cytospins (1.0%–12%) remained higher than those labelled in cell suspension (0.1%–7%), suggesting that a considerable proportion (0.8%–5.2%) of cells expressed cCD3 but no mCD3. These findings were confirmed by double-colour IF staining using a CD3 MAb of IgM class (T10B9) in suspension in combination with one of IgG_1 class (UCHT1) on cytospins. In the earliest samples at the 7th to 9th weeks no $cCD3^+$ cells had mCD3 ($cUCHT1^+$, $mT10B9^-$), while in those from the 11th to 20th weeks 9%–68% of $cCD3^+$ cells expressed mCD3 ($cUCHT1^+$, $mT10B9^+$). Thus T-lineage cells of $cCD3^+$, $mCD3^-$ phenotype persist in fetal liver samples taken after the 11th gestational week, i.e. even after the emergence of $mCD3^+$ lymphocytes (Fig. 1). From these findings we conclude that immature T-lineage cells appear in the fetal liver prior to the emergence of the thymus and occur together with more mature cells after the establishment of the thymic gland.

The phenotypic analysis of $cCD3^+$, $mCD3^-$ cells taken at 7–9 weeks of age showed that >98% were $CD7^+$ ($RFT2^+$), $CD45^+$ ($2D1^+$) and $D8^{+/-}$ ($RFT8^{+/-}$) without any detectable staining with CD1 (Na1/34), CD2 (RFT11), CD5 (RFT1) or CD4 (Leu3). Cytoplasmic CD^{3+} cells were also negative with CD16

(Leu11b), anti-HLA-DR (RFDR1), CD10 (RFAL3) and did not show nuclear TdT staining, although other $CD3^-$ cells including B-cell progenitors were labelled by the same reagents in all the samples studied. The majority (>90%) of $CD7^+$ ($RFT2^+$) cells were $cCD3^+$. These findings confirm the immaturity of these precursors.

In the following experiments the TCRβ chain expression was studied using βF1 in fixed cytocentrifuge preparations. In the liver samples obtained at weeks 7–9 $cCD3^+$ cells failed to express TCRβ chains ($\beta F1^-$). The $\beta F1^+$ cells first appeared after the 11th gestational week and then persisted (Fig. 1). Double-colour IF showed that $\beta F1^+$ cells acquired mCD3 only gradually. In samples taken at weeks 14–16 15%–32% of $\beta F1^+$ cells were still $mCD3^-$. In samples at weeks 17–20 virtually all (>98%) $\beta F1^+$ cells were also $mCD3^+$.

The proliferative activity of $CD3^+$ cells was investigated using a MAb Ki67 which identifies a nuclear antigen expressed during the G_1, S, G_2 and M phases of the cell cycle [29]. In the fetal liver at weeks 7–9 the proportion of $CD3^+$ cells in cycle was 87%–93%. In the 11- to 20-week samples the proportion of $CD3^+$ $Ki67^+$ progressively declined, and only 8%–39% of $CD3^+$ cells were $Ki67^+$ (Fig. 1). This proliferative activity was seen exclusively in the $cCD3^+$, $mCD3^-$ population, for <5% of $mCD3^+$ cells were $Ki67^+$. However, in the 14- to 16-week samples a significant proportion (10%–25%) of $\beta F1^+$ cells were $Ki67^+$, indicating that some $TCR\beta^+$, $mCD3^-$ cells are still in cycle. These findings are compatible with the possibility that $cCD3^+$ cells proliferate locally in the liver and may even develop TCRβ positivity during this proliferative cycle in situ.

II. TCR$\alpha\beta$ Expression in the Fetal and Infant Thymus

In the 15 thymus samples studied from gestational weeks 10.5–20 a high proportion of thymocytes (91%–98%) were $cCD3^+$ ($UCHT1^+$), but only 49%–72% of cells were labelled by βF1, and 19%–42% were $mCD3^{++}$. Double-labelling experiments indicated that, similarly to the fetal liver population, 17%–46% of $cCD3^+$ cells lacked TCRβ chains identifiable by the βF1 MAb. Most of these $cCD3^+$, $\beta F1^-$ cells were blasts with a high nuclear/cytoplasmic ratio and nucleoli. On the other end of the spectrum, the reactivity of WT31, detecting an epitope formed by the assembled $\alpha+\beta$ chain, was seen on 67%–91% of $mCD3^+$ cells. These cells were regarded as maturing T-lymphoid cells [27a].

In the thymocytes obtained at 10.5–18 weeks of gestation nuclear TdT was absent, as reported previously [30]. Nevertheless, positive TdT staining appeared in the four samples studied at the latest gestational periods taken at the 19th to 20th weeks. In these samples TdT^+ cells represented 53%–61% of the total thymocytes, and double-colour IF showed that the large TdT^+ blasts comprised particularly high proportions (31%–80%) of $cCD3^+$, $\beta F1^-$ putative precursor cells. The TdT positivity was absolutely restricted to the $cCD3^+$ cells, of which 67%–79% were $\beta F1^+$, but no TdT^+ cells expressed strongly WT31 among the more than 10^4 analysed [27a].

In seven samples of infant thymus five main differentiation-related markers such as TdT, cCD3, intracellular TCRβ chains (βF1), membrane-associated TCR$\alpha\beta$ (WT31) and mCD3 were studied. Four major categories of cells could be distinguished on the basis of their phenotype defined by double and triple markers (Fig. 2). Firstly, 18% (range 8%–26%) of cells were TdT^+ and expressed cCD3 only, without any detectable TCRβ ($\beta F1^-$). The majority of TdT^+ blasts (60%–80%) were amongst these $\beta F1^-$ cells (category I). The rest of TdT^+ cells (42%; range 22%–62%) were $\beta F1^+$. These were $WT31^{+/-}$ or $WT31^-$ and showed the morphology of typical small cortical thymocytes (category II). With triple-marker analysis a population

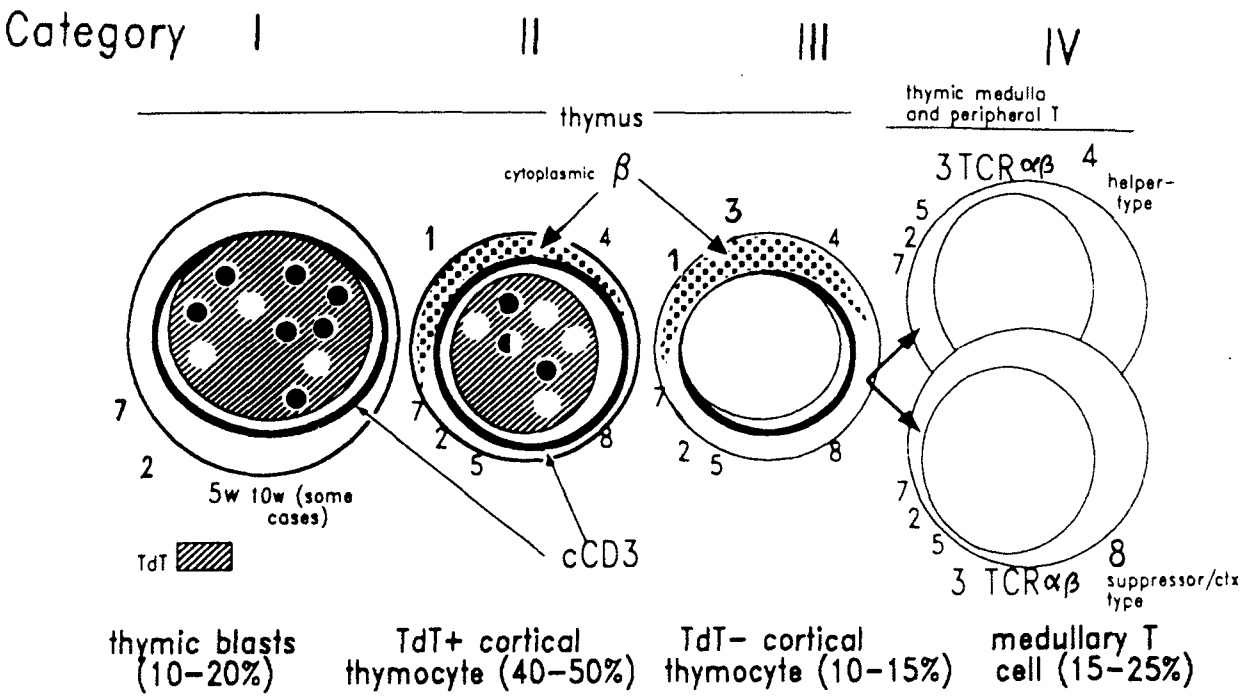

Fig. 2. Stages of T-cell differentiation defined by MAb to TCR associated molecules and TdT. CD3 expression in the cytoplasm (cCD3) and in the membrane (mCD3) is shown by *continuous circles*. The TdT labelling of the nucleus is depicted by a *mesh*. Cytoplasmic TCRβ chains are shown by *small dots*. Cells of categories I and II have a high proliferative activity, indicated by nuclear Ki67 positivity (*white dots; one dot*, 10% positive cells) and bromodeoxyuridine incorporation (*black dots; one dot*, 10% positive cells). The majority of T-ALL show features of proliferating category I (TdT^+, $cCD3^+$, $\beta F1^-$) and category II (TdT^+, $cCD3^+$, $\beta F1^+$) or asynchronous development of TCR molecules [27a]

of $\beta F1^+$ cells (12.5%; range 3%–30%) could be identified which were TdT^- but still lacked strong WT31 (category III). Finally, 23% (range 17%–27%) of cells had features of mature T cells, signified by the membrane expression of assembled TCR$\alpha\beta$ as detected by WT31 (category IV). The intensity of staining with $\beta F1$ on cells of categories III and IV was stronger than that observed on TdT^+ category II thymocytes [27a].

In these infant thymic suspensions, 42% (range 28%–52%) $mCD3^{++}$ cells were seen, including the $WT31^+$ population (category IV) together with most of the TdT^-, $\beta F1^+$ cells (category III). In addition, a few TdT^+ cells (2%–27%) were also weakly $mCD3^+$. These results, taken together, suggest that the insertion of CD3 into the membrane precedes the assembly of TCR$\alpha\beta$ as detected by WT31. Although WT31 binding was visualized with a very sensitive detection method, the immunogold second layer, these findings do not exclude the possibility that the cell type referred to as category III already expresses, in addition to mCD3, small amounts of TCRβ and/or TCRα on its surface. This possibility will need to be further investigated with additional MAbs to TCRβ and TCRα chains.

III. Reactivity of TCRδ-1 in Fetal and Post-natal Tissues

Fifteen samples of fetal liver (7–20 weeks of gestation) and seven samples of fetal thymus (10.5–20 weeks of gestation) were investigated for the expression of TCRδ chain in cell suspension, cytocentrifuge preparations and in cryostat section. No TCRδ-1^+ cells were seen in the liver samples. Very low proportions (0.02%–0.7%) of TCRδ-1^+ cells were observed in the cytospins of fetal thymus, irrespective of whether these were TdT^- or TdT^+, i.e. before or after the 18th week of gestation. The rare TCRδ-1^+

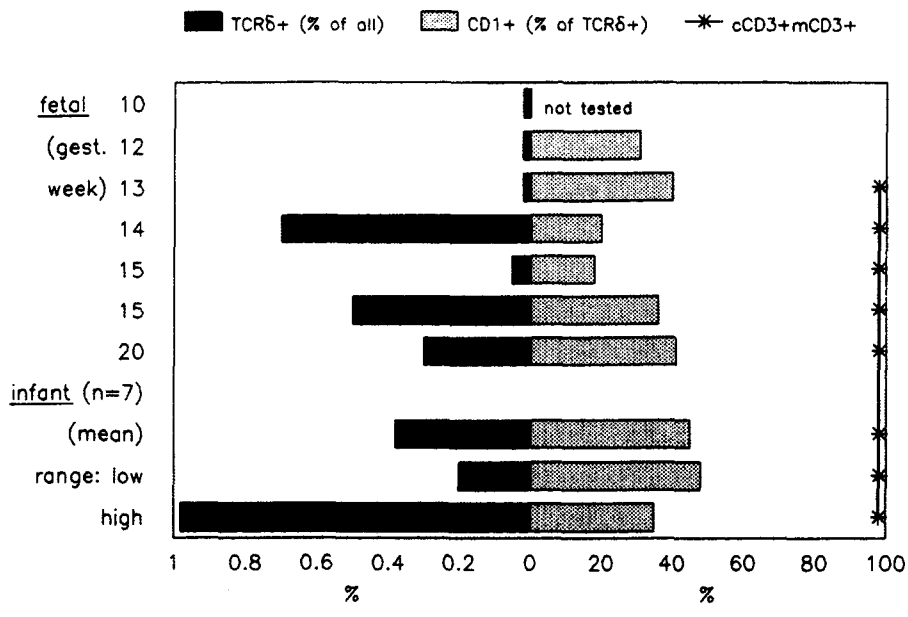

Fig. 3. Expression of TCRδ chains in fetal and infant thymus. TCRδ-1$^+$ cells are invariably CD3$^+$, class II$^-$ and TdT$^-$, but the thymic origin of a proportion of these cells is indicated by CD1 positivity [27a]

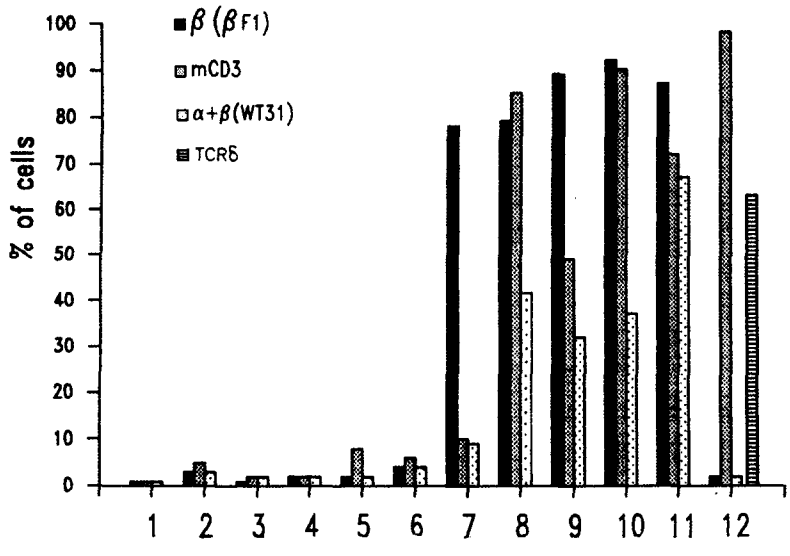

Fig. 4. Heterogeneous expression of TCR proteins in T-ALL. Half of the cases illustrated are βF1$^-$ with few residual normal T cells (TdT$^-$) that are βF1$^+$. Cases 7–11 show βF1 positivity, and in cases 8–11 some of these blasts are also WT31$^+$. Such TdT$^+$, WT31^{++} double-stained cells are not found amongst normal thymocytes. In case 12 mCD3 is associated with TCRδ chains [27b]

thymic cells together with the controls from selected donors with high proportions (9%–13%) of TCRδ-1$^+$ cells were, however, intensely labelled. In infant thymus 0.1%–0.8% cells were TCRδ-1$^+$. Similarly low values (0.1%–0.9%) were observed in 8/10 adult thymus samples, but in 2/10 samples the proportion of TCRδ-1$^+$ cells was somewhat higher – 3% and 4%. These thymic TCRδ-1$^+$ cells were invariably TdT$^-$, class II$^-$ and CD3$^+$. The thymic origin of a proportion (18%–59%) of these cells was indicated by their expression of CD1 (Fig. 3) [27a].

IV. TCR Protein Expression in Malignant T Cells

The 20 cases of T-ALL studied had rearrangements of the TCRβ and TCRγ genes, and contained >70% strongly CD7$^+$ and TdT$^+$ blasts. Cytoplasmic CD3 was detected by UCHT1 in 75%–99% of blasts in all cases studied. These T-ALL cases fell into two categories on the basis of their βF1 positivity, as illustrated by representative samples in Fig. 4. In 12 samples <5% blasts were βF1$^+$ (see e.g. cases 1–6). These βF1$^-$ cases were also WT31$^-$ (<5%) and pre-

dominantly CD1$^-$, CD4$^-$, CD8$^-$. In eight cases of T-ALL 78%–92% βF1$^+$ blasts were detected (see e.g. cases 7–11), and four of these had mCD3 on 49%–85% of blasts and WT31 positivity of 32%–67% of blasts (see e.g. cases 8–11). In the same subgroup of T-ALL some blasts were also CD1$^+$ (5%–63%), and large proportions of blasts (60%–92%) exhibited CD4 and/or CD8 antigens. The same samples were reinvestigated with the TCRδ1 MAb. In two βF1$^-$ cases TCRδ was detected in 48% and 63% of T-ALL blasts (see e.g. case 12). These TCRδ^+ T-ALL were the only tow cases in our series expressing mCD3 without WT31 positivity [27b].

Finally, the lineage specificity of βF1, WT31 and TCRδ1 was investigated in non-T acute leukaemias. Ten cases of common ALL with IgH gene rearrangements were analysed, but none of these showed TCR chains on the membrane or in the cytoplasm in spite of the rearrangements of both TCRγ and TCRβ in two cases, TCRγ in another two cases and TCRβ alone in one more case. All cases of acute myeloblastic leukaemia and of B-cell chronic lymphocytic leukaemia tested showed no positivity with cCD3, βF1, WT31 and TCRδ1 [27b].

D. Discussion

It is known that haemopoietic progenitors reside in the fetal liver from the 5th gestational week, even before they appear in the BM around the 10th week [31]. It is also documented that the fetal liver contains TdT$^+$, CD10$^+$ and cμ^+ B cell progenitors between weeks 11 and 26 [32–35]. In this study we have first investigated the possibility that T-cell progenitors might also be present in the liver in these early stages of development before the formation of the thymus, which occurs at around the 10th week.

The expression of CD3 molecules in the perinuclear area is an early event in T-cell differentiation, and the detection of cCD3 is also a reliable marker for T-cell commitment in T-ALL while other leukaemias of B and myeloid origin are invariable cCD3 negative [21, 22, 24]. Immature cells of the T lineage could indeed be identified in the fetal liver by virtue of their cCD3 positivity, and such precursors appeared to encompass two phases of successive development steps. First, at the 7th to 9th gestational weeks cCD3$^+$ cells were observed with no identifiable mCD3 or β chain (βF1$^-$). These cells expressed CD7 and CD45 without class II or nuclear TdT, and without any other known features of cortical thymocytes such as CD1 and CD4 with CD8. The next cell type, however, carried TCRβ and mCD3, and such cells could be seen from the 11th week onwards. In the samples from weeks 14–16 the double-labelling experiments have identified a few cells showing only TCRβ (βF1$^+$) without mCD3. There are two possible explanations for this finding: cCD3$^+$ cells may undergo development in the liver in situ, resembling the features of T-cell colony-forming cells in vitro [36]. Alternatively, cCD3$^+$ cells may seed to the thymus from where they rapidly disseminate, and some may return to the liver as both "transitional" (TCRβ^+, mCD3$^-$) and more mature (TCRβ^+, mCD3$^+$) cells [27a].

The investigation of the proliferative capacity of these liver-borne immature T cells may give some tentative clues to distinguish between these two possibilities. In the early liver samples (7th to 9th weeks) the majority (>90%) of cCD3$^+$ cells are Ki67$^+$ cells within their cell cycle while the mCD3$^+$ liver lymphocytes are virtually all Ki67$^-$ (>95%). In the 14- to 16-week samples the βF1$^+$ population includes 10%–25% of Ki67$^+$ cells, indicating that the TCRβ^+, mCD3$^-$ cells are in cycle [27a]. Thus, at least some of the mCD3$^+$ cells might originate locally from these immature T cells. Lobach et al. have already described CD7$^+$, CD45$^+$ cells in the sinusoids of fetal liver samples and the perithymic parenchyma at the 7th week [37]. In their study the

$CD7^+$ cells appeared to be $CD3^-$ when tested with the Leu4 MAb. The discrepancy between these and our findings might be due to technical difficulties in detecting low antigen levels in fetal tissues.

Other cell types with the putative prothymocyte features have also been described, and future studies may indeed reveal that among these are the cells which most efficiently home to and proliferate in the thymus. Nevertheless, the question of these cells' commitment to the T-cell lineage prior to their arrival to the thymus remains unanswered. Hokland et al. reported that after the seeding of $CD10^+$ fetal BM cells onto irradiated thymic cells in vitro lymphoid cells of $CD1^+$, $CD4^+$, $CD8^+$ phenotype develop in the presence of interleukin-2 [38]. Van Dongen et al. have characterized a rare $CD7^+$, TdT^+, Class II^+ cell type in the adult BM and suggested that it might fit the description of prothymocytes [39]. These features are also seen in a group of acute non-lymphoid leukaemias which show no signs of TCRβ or IgH gene rearrangements [40]. In contrast, the detection of cCD3 can be taken as a reliable expression of T-cell commitment [21, 22, 24, 41]. This is demonstrated in malignant disease such as T-ALL as well as in the progenitor cell line KG-1. This line lacks CD3 antigens and exhibits a germline configuration of the TCRβ genes together with myeloid antigens; a subclone of this cell line, KG-1a, however, expresses cCD3, TCRβ gene rearrangement and CD7, paralleled by the disappearance of the myeloid associated antigens [42]. These observations support the view that CD3 antigens accumulate in the perinuclear area at the earliest stages of T-cell differentiation and imply that $cCD3^+$ cells in the fetal liver include a class of TdT^- prothymocytes.

These data taken together suggest a model of human T-cell ontogeny in which $cCD3^+$, $CD7^+$, TdT^- T-cell progenitors originate and proliferate in the liver before thymic generation. The capacity of these progenitors to colonize thymic epithelium needs to be investigated. The question of a second TdT^+ prethymic cell also remains open because we find that $cCD3^+$, TdT^+ cells are restricted to the thymus and are absent from the fetal liver as well as in the fetal and adult BM.

In the infant thymus four major subpopulations have become identifiable with the help of reagents to TCR-associated proteins [27a]. The large TdT^+ blasts show progenitor features, such as a high proliferative activity, and expression of cCD3 [20–22, 42]. Many of these thymic cells lack cytoplasmic TCRβ chains (category I). In these $mCD3^-$, $CD1^-$ blasts a germline configuration of the TCRβ genes was reported [21], but the DNA analysis in such a small polyclonal population presents considerable technical difficulties. The majority of TdT^+ cells are smaller and heterogeneously exhibit mCD3, TCRβ, CD1, CD4 and CD8 antigens. The expression of TCRβ chains appears to be initiated at this cortical thymocyte stage (category II). The gradual change into the third cell category is signified by the loss of TdT and the presence of cells which contain TCRβ chains but do not as yet exhibit a fully assembled TCR$\alpha\beta$ that would be detectable by WT31 (category III). This minority population is similar to the TdT^- pre-B cells seen in the BM which show cytoplasmic μ heavy chains without membrane Ig. These findings also confirm observations in murine neonatal thymus where free cytoplasmic β chains are synthesized at high rate but are rapidly degraded in thymocytes with no detectable surface receptor [43]. Finally, the α chains are produced, and the assembly of membrane bound TCR is initiated. Our study suggests that the insertion of CD3 into the membrane might precede that of the fully assembled TCR$\alpha\beta$ complex.

On the basis of studies indicating the early rearrangement and expression of TCR$\gamma\delta$ genes in T-cell ontogeny [4–6, 9, 11], we anticipated that a particularly high proportion of $TCR\delta^+$ precursors

might be detectable in the early fetal liver and thymus, perhaps coinciding with the TdT^- stage of development. The results do not confirm this hypothesis because the output of $TCR\delta^+$ cells remains low at the time of the highest TdT^- proliferative activity among $cCD3^+$ cells in the fetal liver and thymus [27a].

Studies in rodents [10] and avians [11, 12] have demonstrated that the development of at least a proportion of the $TCR\gamma\delta$ lineage cells occurs in the thymus. The expression of CD1 in 18%–59% of $TCR\delta1^+$ fetal and post-natal thymic cells, shown in our investigation, provides evidence for such a process. Despite CD1 positivity, we could not find $TCR\delta1^+$ thymic cells with nuclear TdT. There are two possible explanations for this observation. The generation of diversity of the $TCR\delta$ chains might be totally TdT independent, or TdT might be lost as soon as the synthesis of $TCR\delta$ chains is initiated, similar to the events occurring during B-cell development: in B-cell progenitors TdT expression ceases with the accumulation of μ heavy chains [44]. The possibility that TdT does contribute to the generation of diversity of $TCR\delta$ is also suggested by the observation that N-region nucleotide addition in the $TCR\delta$ gene is seen in murine thymic clones when these are derived from TdT^+ post-natal samples, but not when derived from TdT^- fetal thymic samples [9].

The paucity of $TCR\delta1$ positivity on thymocytes is surprising in view of the fact that some cases of T-ALL successfully synthesize $TCR\delta$ [27b] and $TCR\gamma$ [45] chains. The comparative analysis of normal and leukaemic thymic cells suggests that most cases of T-ALL reflect the features of the two earliest stages of TdT^+ development such as $cCD3^+$, $TCR\beta^-$ (category I) and $cCD3^+$, $TCR\beta^+$ (category II). Nevertheless, in a substantial number of samples asynchronous, aberrant expressions of TCR receptor proteins are also seen. An example is the combined presence of both TdT and bright WT31, a phenomenon not seen in normal cells [27a, b].

A further point of interest is as follows. In 6 of the 20 T-ALL cases studied, blasts expressed mCD3. This was associated with membrane $TCR\alpha\beta$ chains in four cases and with $TCR\delta$ chains in the remaining two. The fact that both patients with $TCR\delta^+$ T-ALL had enlarged mediastinal mass suggests thymic involvement and indirectly confirms the occurrence of a thymic development of $TCR\gamma\delta$ cells in humans, as already indicated by the CD1 expression on $TCR\delta1^+$ thymic cells. In addition, the TdT positivity of the blasts in the two $TCR\delta^+$ T-ALL cases further demonstrates the asynchronous phenotypic expression of leukaemia [27b). It remains to be investigated whether this heterogeneity of T-ALL is reflected in their clinical course and response to treatment.

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